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<b>(21) International Application Number:</b> PCT/US97/03465 <b>(22) International Filing Date:</b> 21 March 1997 (21.03.97)  <b>(30) Priority Data:</b> 60/013,818                      21 March 1996 (21.03.96)                      US  <b>(71) Applicant (for all designated States except US):</b> DANA-FARBER CANCER INSTITUTE [US/US]; 44 Binney Street, Boston, MA 02115 (US).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> TEICHER, Beverly, A. [US/US]; 135 Hunting Road, Needham, MA 02194 (US).  <b>(74) Agents:</b> EISENSTEIN, Ronald, I. et al.; Dike, Bronstein, Roberts & Cushman, L.L.P., 130 Water Street, Boston, MA 02109 (US).		<b>(81) Designated States:</b> AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> USE OF ALLOSTERIC HAEMOGLOBIN MODIFIER FOR TREATING SOLID TUMORS AND INHIBITING TUMOR METASTASES  <b>(57) Abstract</b>  A method for treating neoplastic growth and/or metastases is described. This involves using allosteric effectors, preferably fibrin acid derivatives. In one embodiment, these compounds are used in a subject having a tumor experiencing rapid growth and at risk for metastases. In another embodiment, these compounds are used with a second antineoplastic agent such as radiation or a chemotherapeutic agent.		

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USE OF ALLOSTERIC HAEMOGLOBIN MODIFIER FOR TREATING SOLID TUMORS AND  
INHIBITING TUMOR METASTASES

BACKGROUND OF THE INVENTION

The present invention is directed to new methods for treating neoplastic growth and also to new methods for inhibiting the formation of tumor metastases.

5 Solid tumors can exist in an asymptomatic state for many years. Typically the tumor is relatively small and can readily escape detection. However, a series of events associated with angiogenesis can trigger the growth of the tumor. The tumor can then grow to many times its original size. This growth can damage adjacent  
10 tissues and organs. Moreover, the neoplastic growth does not remain localized, instead neoplastic cells can shed from the original tumor and spread resulting in tumor formation at distant sites.

For most patients, cancer is or becomes a systemic  
15 disease. The common solid tumors frequently develop a primary tumor (bulk disease) which sheds malignant cells into the blood and lymph circulation. It is these shed cells that are responsible for the spread of the malignancy. A small fraction of these circulating  
20 malignant cells initiate tumor growth at sites within the host distant from the primary tumor (which is referred to as metastases). Treatment of malignant disease requires

eradication of tumor cells at all sites. However, treatment of multiple sites poses significant obstacles. Thus, often an initial goal of anticancer therapy when metastasis has not yet occurred or is below the level of clinical detection, is control of the primary tumor.

A second goal if eradication of the tumor is unlikely, often is to try to slow down (inhibit) the rate of tumor growth and/or metastases. Trying to achieve these goals involves a number of different treatment regiments.

For example, one typically uses an anticancer agent. Such an agent is one that will selectively kill a neoplastic cell. These types of agents include cytotoxic agents, agents that stimulate the immune system to attack the neoplastic cell, anti-angiogenic agents, anti-antihormonal agents, etc.

Currently, radiation therapy is the primary non-surgical treatment modality used to achieve local control of tumors. However, while radiation preferentially kills neoplastic cells, it can also damage normal growing cells that are exposed to the radiation. Further, the efficacy of radiation therapy is extremely dependent upon the oxygen content of the tumor. For instance, it is well-established that oxygen is rapidly metabolized by cells and, therefore, in tissues it has a limited diffusion

distance from vasculature (Gatenby et al., 1988; Gullino, 1975; Hasegawa et al., 1987; Jain, 1988; Sevick and Jain, 1989; Song et al., 1987; Vaupel et al., 1987; Vaupel et al., 1981).<sup>1</sup> Those regions that do not contain  
5 sufficient oxygen are considered hypoxic regions. Such regions of hypoxia have been demonstrated in many solid tumor model systems (Gullino, 1975; Hasegawa et al., 1987; Jain, 1988; Sevick and Jain, 1989; Song et al., 1987; Vaupel et al., 1987) and in human solid tumors  
10 (Vaupel et al., 1989) by several different methods.

Such regions of hypoxia help to protect tumor cells from damage by cytotoxic therapies that are directly and/or indirectly oxygen dependent. For example, hypoxia can lead to therapeutic resistance through: 1) direct  
15 effects due to a lack of O<sub>2</sub> which some drugs and radiation require to be maximally cytotoxic; 2) indirect effects via altered cellular metabolism which decreases drug cytotoxicity; and 3) enhanced genetic instability which can lead to more rapid development of drug  
20 resistant tumor cells.

Consequently, methods of enhancing delivery of oxygen can be a useful way for improving the oxygenation of solid tumor by altering the gradient of oxygen as it

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<sup>1</sup> Full citations to the references appear immediately before the claims.

is absorbed from the vasculature and distributed into the tissue (Teicher and Herman, 1993; Teicher et al., 1992; Teicher et al., 1994b; Teicher et al., 1993a; Teicher et al., Teicher et al., 1993b; Teicher et al 1994c; Vitu-  
5 Loas et al., 1993). Oxygen delivery to tumor masses should be improved by increasing the release of oxygen from red blood cells (Teicher, 1995). Methods of accomplishing this include the use of oxygen delivery agents and hemoglobin modifiers to selectively change the  
10 hemoglobin's oxygen affinity.

For example, in addition to the quantity of hemoglobin (Hb) available for oxygen transport, the amount of oxygen released at the tissues is important. The amount of O<sub>2</sub> released strongly depends on the  
15 position of the Hb-O<sub>2</sub> dissociation curve (Hb affinity for O<sub>2</sub>). Alterations in the position of the Hb-O<sub>2</sub> dissociation curve have been shown in preclinical investigations to influence the radiation response of animal tumors (Hirst and Wood, 1987; Hirst et al., 1987; Siemann et al., 1978; Siemann et al., 1979; Siemann and  
20 Macler, 1986). Reductions in the Hb affinity for O<sub>2</sub> can sensitize tumors to radiation (Hirst and Wood, 1987; Hirst et al., 1987; Siemann et al., 1979; Siemann and Macler, 1986). Chemical agents or physiologic  
25 manipulations aimed at sensitizing tumors by shifting the

Hb-O<sub>2</sub> dissociation curve to a position that favors O<sub>2</sub> off-loading have been evaluated (Hirst and Wood, 1987; Hirst et al., 1987; Siemann et al., 1979; Siemann and Macler, 1986).

5           For instance, Siemann et al. (Siemann and Macler, 1986) demonstrated that the acute elevation of 2, 3-diphosphoglycerate (2,3 DPG) levels in the blood of KHT sarcoma bearing mice significantly enhanced the response of the tumors to single doses of radiation. This  
10       improvement was primarily the result of a reduction in the fraction of hypoxic tumor cells from about 15% to about 3%. These results demonstrated that the manipulation of erythrocyte 2,3 DPG levels may be an effective approach to improving the tumor response to  
15       radiotherapy. In the early 1980's it was recognized that the antilipidemic fibric acid analogs, clofibrate, bezafibrate and gemfibrozil, decreased the affinity of hemoglobin for oxygen *in vitro* (See. U.S. Patent Nos. 5,290,803; 5,250,701; 5,248,785; 5,122,539 and 5,049,695;  
20       WO 92/20335; Abraham et al., 1983; Perutz and Poyart, 1983; Wooton, 1984). Hirst et al. (1987) was able to show that administration of clofibrate of RIF-1 tumor-bearing mice was able to markedly sensitize the tumor to single dose radiation. Building on this knowledge,  
25       Abraham and colleagues prepared and characterized a



series of fibric acid derivatives that are potent  
allosteric effectors of oxygen binding to hemoglobin and  
that pass freely in and out of red blood cells (Abraham  
et al., 1995; Abraham et al., 1992; Khandelwal et al.,  
5 1993; Wei et al., 1993).

The synthesis of these compounds is disclosed in  
U.S. Patent Nos. 5,290,803; 5,250,701; 5,248,785;  
5,122,539 and 5,049,695 all which are hereby incorporated  
by reference. The use of such allosteric effectors is  
10 expected to enhance the effectiveness of radiation  
therapy and cytotoxic agents by permitting one to lower  
the O<sub>2</sub>-binding affinity of hemoglobin, thereby making  
oxygen more available to the cells. While numerous  
agents have been proposed for treating neoplastic growth,  
15 additional agents are still necessary. Preferably, such  
an agent should be relatively benign to normal cells.

Methods of treatment have also been proposed for  
inhibiting metastasis. For example, it has been known  
that certain anticoagulants, hypolipidemic agents and  
20 related molecules can alter tumor growth and especially  
tumor spread or metastasis.

Early theories regarding the antimetastatic actions  
of these molecules centered on the survival of tumor  
cells in the circulating blood and the ability of these  
25 tumor cells to extravasate from circulation to implant in



distant tissues (Hayashi et al., 1995; Hilgard, 1984; Pacot et al., 1993). Recently, it has been shown that lovastatin, a naturally occurring hypolipidemic agent that inhibits cholesterol biosynthesis, decreased the number of lung colonies formed when mice were injected intravenously with  $10^6$  B16 melanoma cells and treated with lovastatin (50 mg/kg) 3-times per week beginning on the same days as tumor cell injection (Jani et al., 1995). Lovastatin had no effect on subcutaneous B16 melanoma growth but in cell culture studies decreased the attachment, the motility and the invasion activities of lovastatin treated B16 cells (Jani et al., 1995).

It would be desirable to discover new methods for inhibiting neoplastic growth and/or metastasis. It would be extremely useful to discover such methods where the agents used are less toxic than other agents.

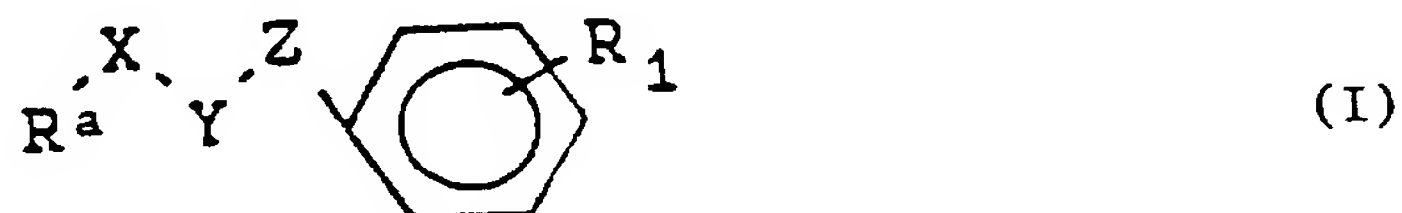
#### Summary of the Invention

We have now discovered a new method for treating neoplastic growth and/or metastasis. This method unexpectedly involves using a class of molecules, fibric acid derivatives, previously proposed as allosteric hemoglobin modifiers. These compounds include those described in WO 92/20335, U.S. Patent No. 5,290,803, U.S. Patent No. 5,250,701, U.S. Patent No. 5,248,785, U.S.

Patent No. 5,122,539, and U.S. Patent No. 5,049,695, which are incorporated herein by reference.

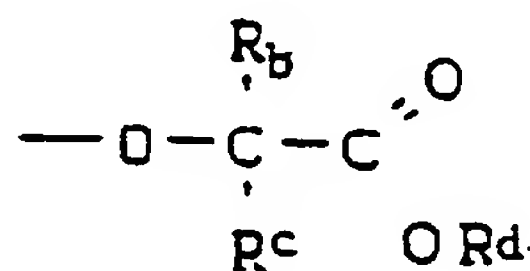
Preferably the compounds have the general structure of formula (I):

5



10 where  $R_1$  is a substituted or unsubstituted aromatic compound, or a substituted or unsubstituted alkyl ring compound, or a substituted or unsubstituted phthalimide compound where X is a carboxyl, Y is a nitrogen and  $R_2$  completes the phthalimide compound by being bonded to  
 15 both X and Y, and where X, Y, and Z are  $\text{CH}_2$ , NH, CO, O or N with the caveat that the X, Y, and Z moieties are each different from one another, and where  $R_1$  has the formula:

20

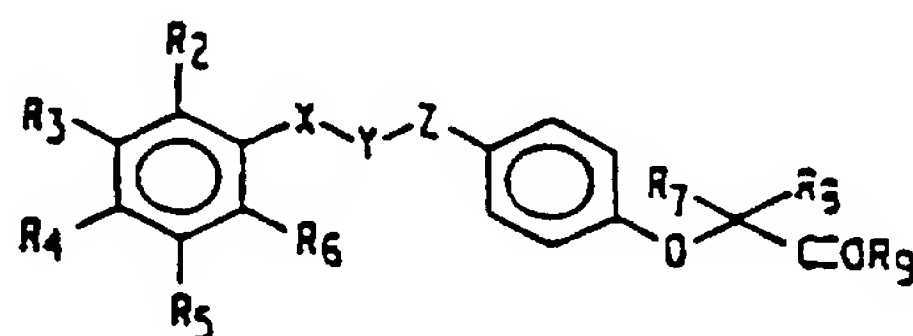


where  $R_1$  can be connected to any position on the phenyl ring, and where  $R_b$  and  $R_c$  are hydrogen, halogen, methyl, or different, or alkyl moieties as part of an

aliphatic ring connecting  $R_b$  and  $R_c$ , and where  $R_d$  is a hydrogen, halogen,  $C_{1-3}$  lower alkyl, or a salt cation.

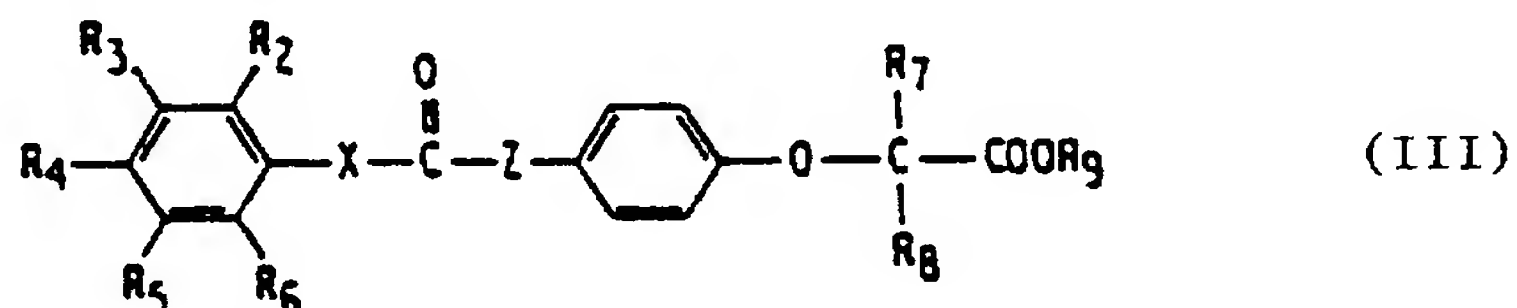
Preferred subsets of compounds may be classified into the following groupings.

- 5           With reference to the general structural formula of Formula (II) the X and Z moieties may be  $CH_2$ , CO, NH or O, and the Y moiety may be CO or NH, with the caveat that the X, Y and Z moieties are each different from one another. In addition,  $R_{2-6}$  are either hydrogen, halogen,
- 10           a substituted or unsubstituted  $C_{1-3}$  alkyl group (up to three carbons in length), or a  $C_{1-3}$  ester or ether and these moieties may be the same or different, or alkyl moieties of aliphatic or aromatic rings incorporating two adjacent  $R_{2-6}$  sites. The  $R_{7-8}$  positions are hydrogen,
- 15           halogen, methyl, or ethyl groups of these moieties may be the same or different, or alkyl moieties as part of an aliphatic (e.g., cyclobutyl) ring connecting  $R_7$  and  $R_8$ . The  $R_9$  position is a hydrogen, halogen,  $C_{1-3}$  lower alkyl such as methyl, ethyl or propyl, or a salt cation such as
- 20           sodium, potassium, or ammonium.

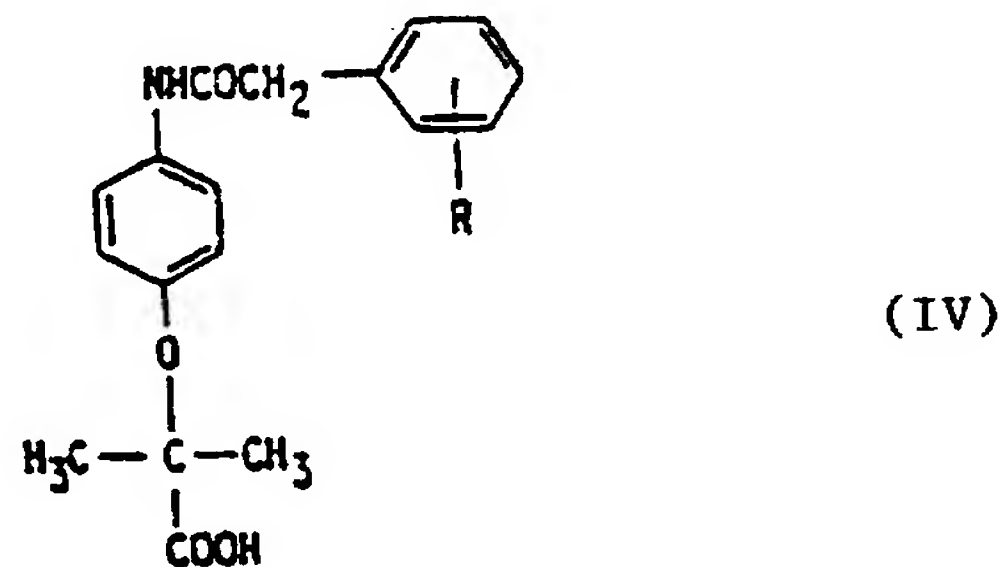


(II)

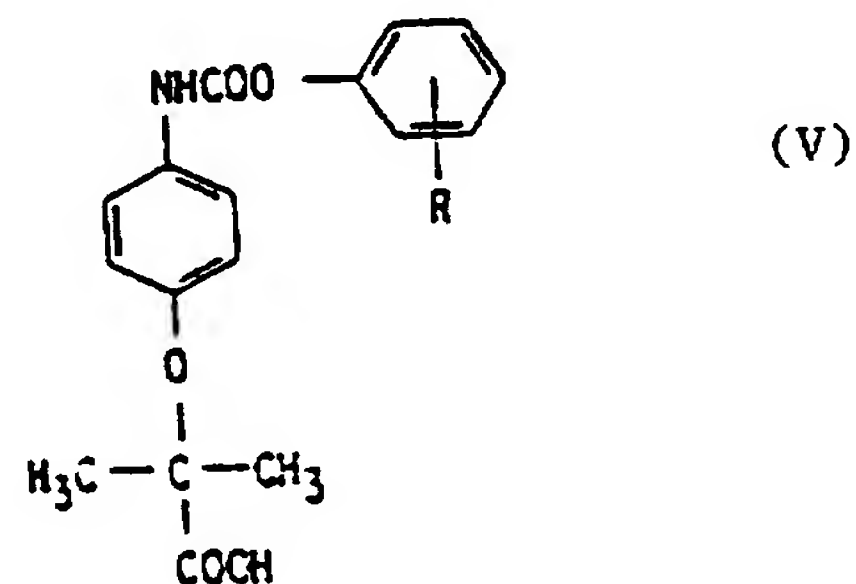
In the first subset of compounds defined in Formula (III) X and Z may each be CH<sub>2</sub>, NH, or O, with the caveat that when X is CH<sub>2</sub>, Z is either NH or O, and when X is NH, Z is either CH<sub>2</sub> or O, and when X is O, Z is NH or CH<sub>2</sub>. The first subset of compounds may conveniently be classified into four groupings as follows:



10      Group I:      2-[4((aryl)acetamido)phenoxy]-2-methyl propionic acid compounds having the general structural formula illustrated in Formula (IV).

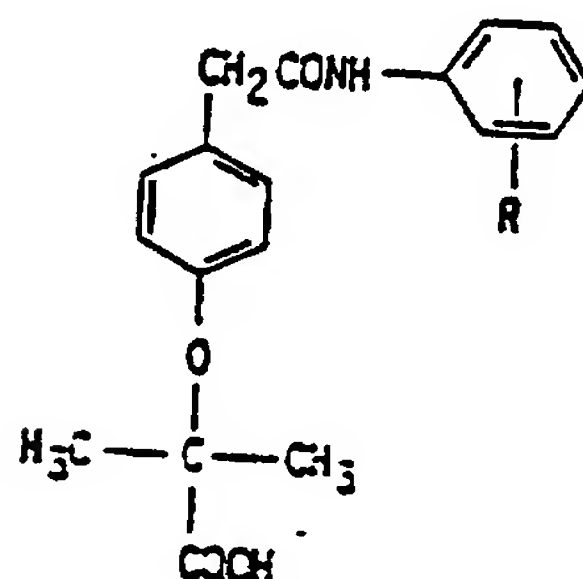


20      Group II:      2-[4-(((aryl)oxy)carbonyl)amino) phenoxy]-2-methyl propionic acid compounds having the general structural formula illustrated in Formula (V).



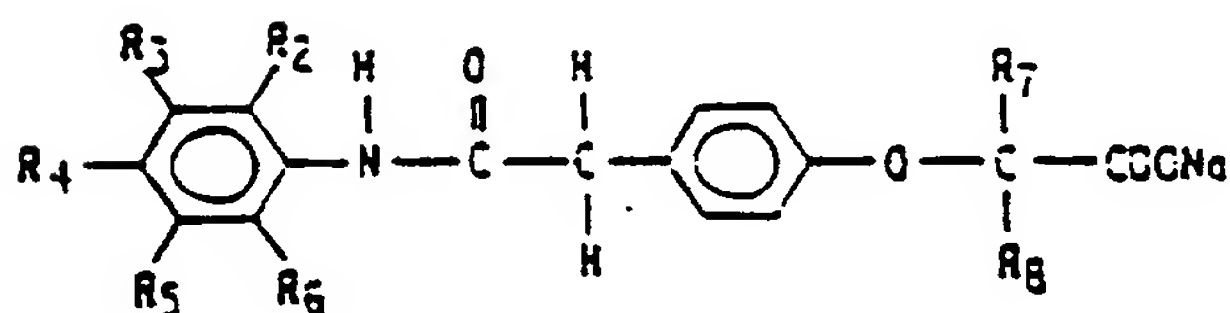
5      Group III:      2 - [4 -  
                              (((aryl) amino) carbonyl) methyl) phenoxy] - 2 -  
                              methyl propionic acid compounds having the  
                              general structural formula illustrated in  
                              Formula (VI and VII).

10



(VI)

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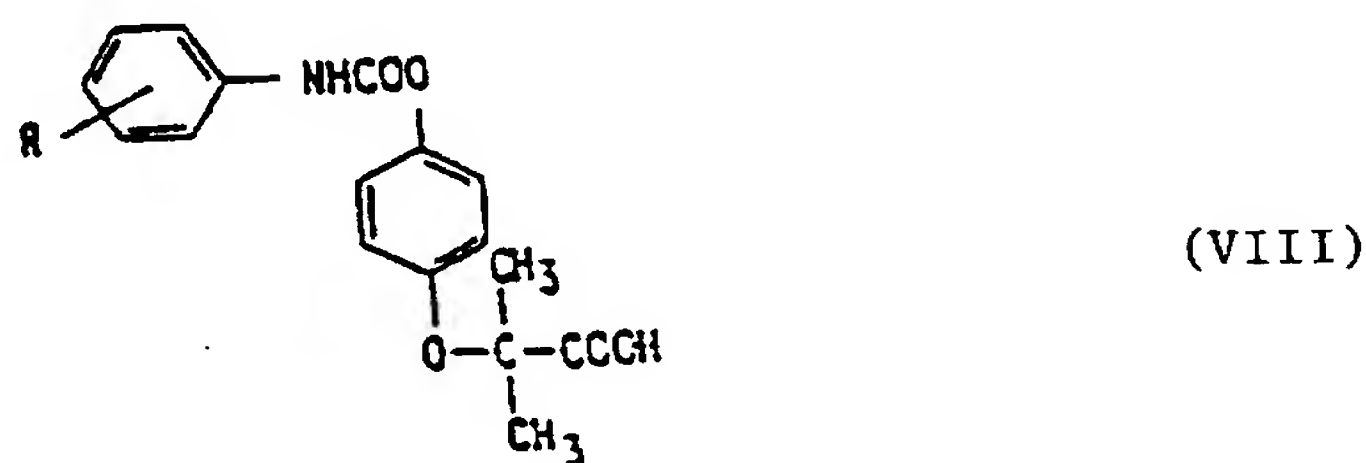


(VII)

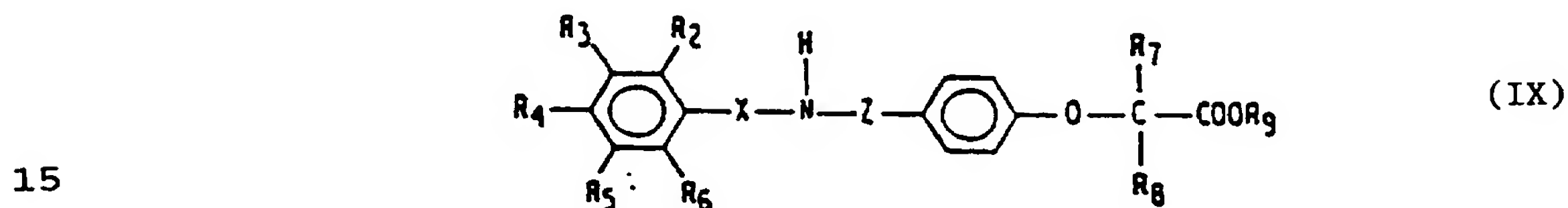
20

Group IV:      2 - [4 - (((aryl) amino) carbonyl) oxy) phenoxy] -  
                              2-methyl propionic acid compounds having

the general structural formula illustrated  
in Formula (VIII).

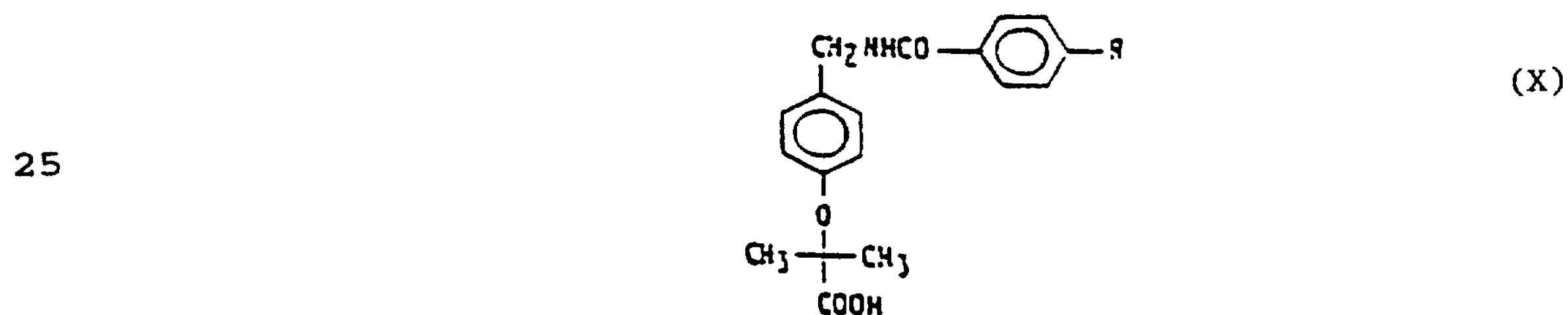


10 In the second subset of compounds defined in Formula (IX)  
X and Z may each be CO or CH<sub>2</sub>, with the caveat that when  
X is CO, Z is CH<sub>2</sub>, Z is CO.



The second subset of compounds may be conveniently  
divided into two groupings as follows:

20 Group V: 2-[4-(((aryloyl)amino)methyl)phenoxy]-2-methyl  
propionic acid compounds having the general structural  
formula illustrated in Formula (X).



Group VI: 2-[4-(((aryl)methyl)amino)carbonyl]phenoxy]-2-methyl propionic acid compounds which are the  
5 subject matter of U.S. Patent application  
07/623,346 to Abraham et al. filed December 7,  
1990.

The use of 2-[4-(((aryl)amino)carbonyl)methyl]-  
phenoxy]-2-methyl propionic acid compounds are more  
10 preferred. The use of the compound where the aryl group  
is a substituted phenyl such as a dimethyl phenyl, e.g.  
2-[4-(((3-5-dimethyl phenyl)amino)carbonyl)  
methyl]phenoxy]-2-methyl propionic acid ( $C_{20}H_{23}NO_4$ )  
(sometimes called RSR-13) is even more preferred. The  
15 use of the compound RSR-13 is still more preferred.

Other preferred compounds include those having the  
same X-Y-Z bridge as RSR-13 (i.e. -NHCOCH<sub>2</sub>-). Preferred  
compounds have the same general structure as RSR-13, but  
differ in their functional group substitution as follows:  
20 RSR-13: 3,5-Dimethyl;  
JP-7: Replacement of gem-dimethyl by cyclopentane ring;  
KDD-86: Mixed methyl (3)-chloro(5);  
RSR-46: Cyclopentyl ring instead of dimethyls;  
RSR-4: 3,5-Dichloro;  
25 GSJ-61: 3-Ethoxy; and



GSJ-60: 4-Ethoxy.

These compounds are less potent allosteric effectors than RSR-13. Potency decreases in the descending order shown:

5 JP-7>KDD-86>RSR-4>RSR-46>GSJ-61.

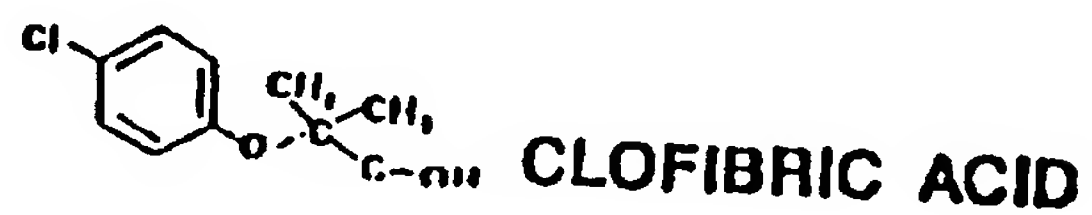
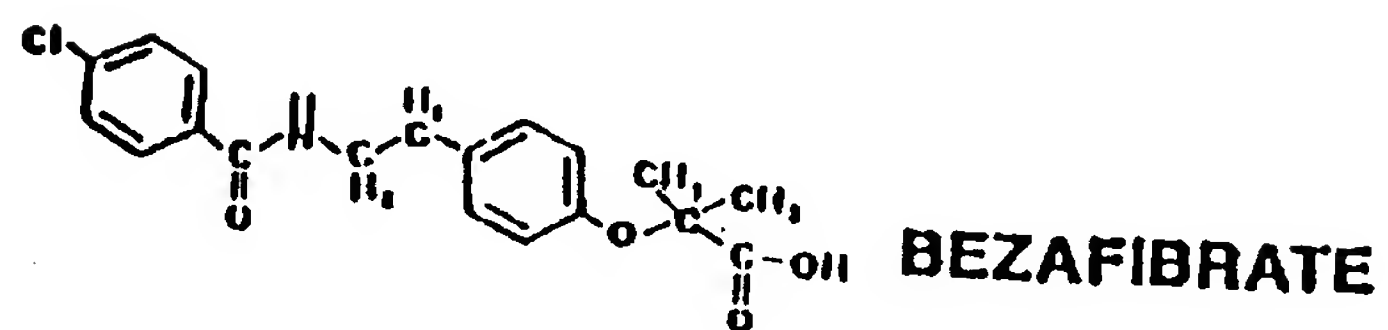
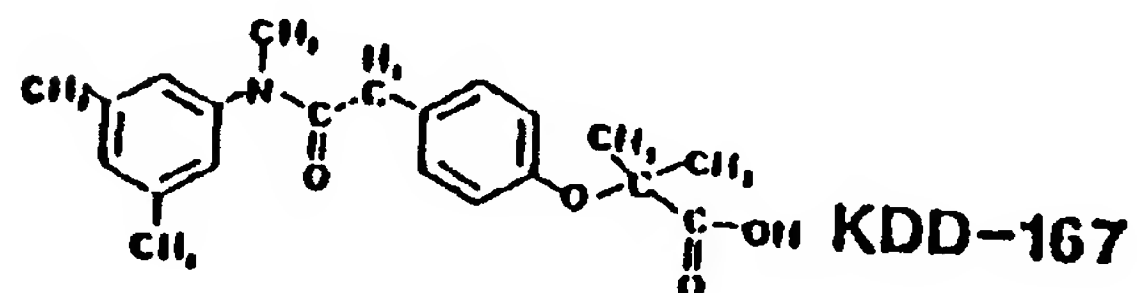
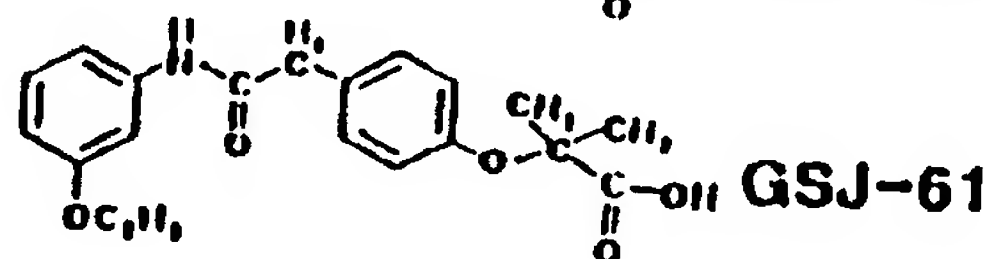
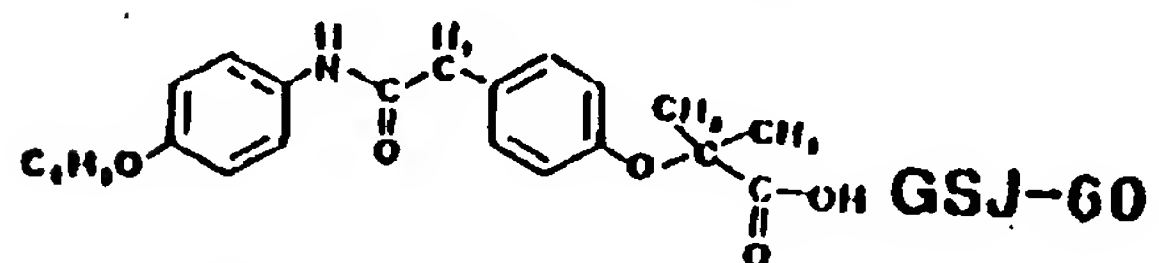
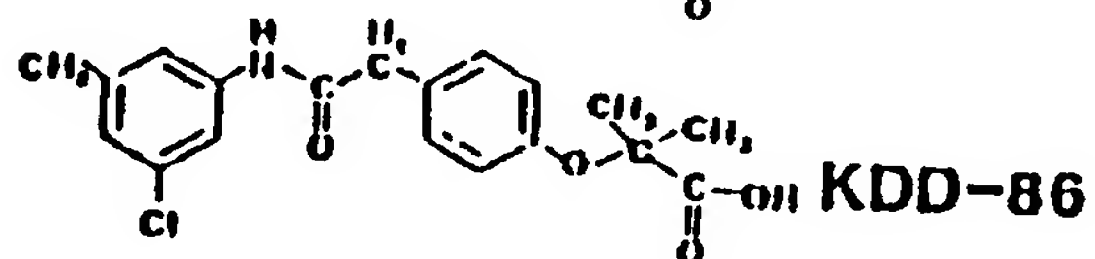
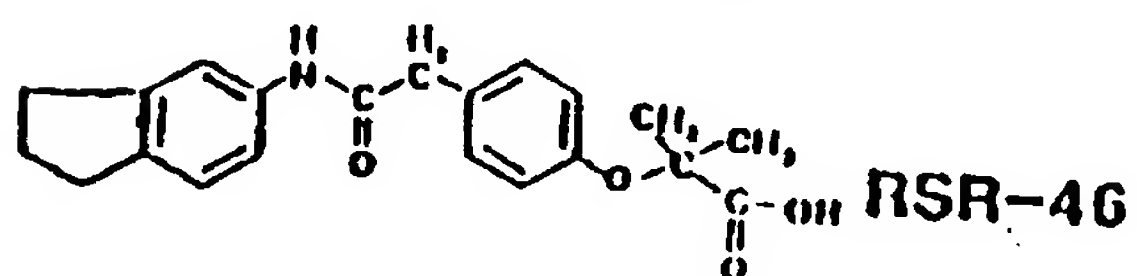
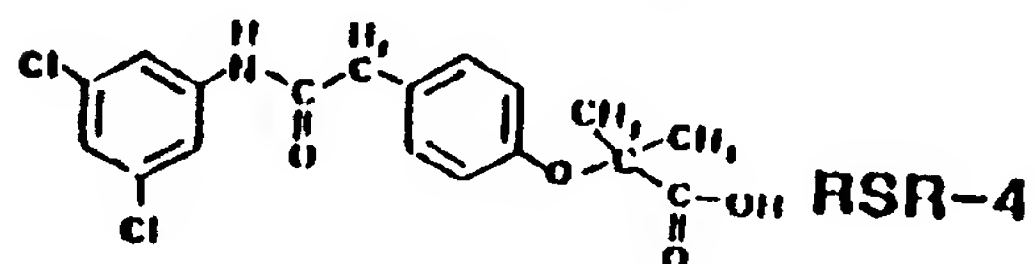
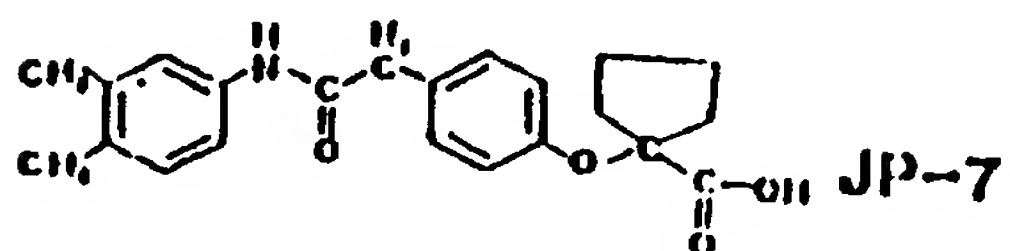
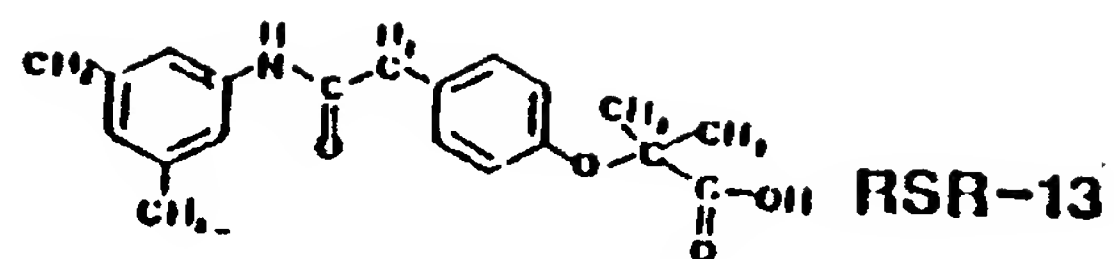
Another group of preferred compounds have a similar structure as RSR-13, but the following bridges:

-CONHCH<sub>2</sub>, -CH<sub>2</sub>CONH-, -CH<sub>2</sub>NHCO-, NCH<sub>3</sub>COCH<sub>2</sub> - and -  
10 NHCONH- (which latter compounds have the structure of  
Formula (I) with the exception that the bridge is X-Y-X,  
where X and Y are defined as above, but both X's are the  
same.

Preferred bridges and functional group substitutions are set forth below:

15 MM 27(-CONHCH<sub>2</sub>) 3,5 Dichloro;  
MM 30(-CONHCH<sub>2</sub>) 3,4,5 Trichloro;  
RSR 35(-CH<sub>2</sub>CONH) 3,5 Dimethyl;  
LS 3,5 (-NHCONH-) 3,5 Dichloro;  
LS 3,4,5 (-NHCONH-) 3,4,5 Trichloro; and  
20 RSR 16 (-CH<sub>2</sub>NHCO-) R=H  
KDA - 167 (-NCH<sub>3</sub>COCH<sub>2</sub>) 3,5 Dimethyl

The structures of some of the preferred compounds and related compounds are set forth below.



Preferably, one administers an effective amount of one of those compounds to a subject such as a mammal, preferably a human, having neoplastic cells, such as a solid tumor or lymphoma. One preferred subject is one  
5 experiencing rapid growth of the tumor and at risk for shedding of malignant cells (i.e. metastasis). Accordingly, one would determine whether a subject is at such risk and thereafter administer the compounds in the present method. Treating a mammal with a solid tumor is  
10 preferred.

Preferably, the compound is administered in conjunction with at least a second antineoplastic agent. Such agents include radiation and chemotherapy agents. These chemotherapy agents preferably include those from  
15 major classes of antitumor agents as exemplified by cyclophosphamide, adriamycin and 5-fluorouracil.

#### Brief Description of the Drawings

Figure 1 shows chemical structures of RSR-13,  
20 bezafibric acid and clofibric acid.

Figure 2 A-D shows histograms showing the oxygen content of the rat 13762 mammary carcinoma as well as the percent of pO<sub>2</sub> readings <5 mmHg and the tumor median pO<sub>2</sub> under each condition: Figure 2A shows air breathing,  
25 Figure 2B shows carbogen (95% oxygen/5% carbon dioxide)

breathing, Figure 2C shows 30 minutes after intravenous administration of RSR-13 (150 mg/kg)/air breathing and Figure 2D shows 30 minutes after intravenous administration of RSR-13 (150 mg/kg) carbogen breathing. Each histogram is based on at least 600 individual  $pO_2$  readings.

Figure 3 is a graph showing the percent of  $pO_2$  readings < 5 mmHg and median  $pO_2$  in the rat 13762 mammary carcinoma over the time course of a 60-minute infusion of RSR-13 (200 mg/kg) and the 30 minutes after completion of the infusion. Data are based on measurements from 6 tumor-bearing animals.

Figure 4 is a graph showing the survival of EMT-6 tumor cells (●) and bone marrow CFU-GM (O) from animals treated with single intraperitoneal injections of RSR-13. Points are the means of three independent experiments; bar are the S.E.M.

Figure 5 is a graph showing the mean number of lung metastases on day 20 from animals injected intravenously with Lewis lung carcinoma cells ( $10^4$ ) on day 0. Animals were untreated (shaded area) or were treated with RSR-13 (100 mg/kg) (●) or RSR-13 (200 mg/kg) (O) for five intraperitoneal injections once per day beginning on days -1, 0, 1, 3, or 7. Points are the means of 15 animals; bar are the S.E.M.

Figures 6A and B show survival of EMT-6 murine mammary carcinoma cells exposed under normally oxygenated (Fig. 6A) or hypoxic conditions (Fig. 6B) to radiation alone (\*) or along with an allosteric effector of hemoglobin for 1 hour prior to, during and for 1.5 hours after radiation delivery. The symbols are: 1) RSR-13 (◐), 2) JP-7 (◑), 3) RSR-4 (◒), 4) RSR-46 (◓), 5) KDD-86 (◔), 6) GSJ-61 (◕), 7) KDD-167 (◖) and 8) GSJ-60 (◗). The data are the means of three experiments.

Figures 7A, 7B, 7C and 7D show survival of EMT-6 murine mammary carcinoma cells exposed to various concentrations of 4-hydroperoxycyclophosphamide (4-HC) (Fig. 7A), melphalan (Fig. 7B), thiotepa (Fig. 7C) or carboplatin (Fig. 7D) for 1 hour alone (\*) or along with an allosteric effector of hemoglobin. The symbols are: 1) RSR-13 (◐), 2) JP-7 (◑), 3) RSR-4 (◒), 4) RSR-46 (◓), 5) KDD-86 (◔), 6) GSJ-61 (◕), 7) KDD-167 (◖) and 8) GSJ-60 (◗). The data are the means of three experiments.

Figures 8A and 8B show survival of EMT-6 murine mammary carcinoma cells from tumors treated in vivo and bone marrow CFU-GM from the same animals after treatment of the tumor-bearing animals with single doses (100, 300 or 500 mg/kg) of the allosteric effectors of hemoglobin by intraperitoneal injection. The symbols are: 1) RSR-13

















() , 2) RSR-4 () , 3) RSR-46 () , 4) GSJ-61 () , 5) GSJ-60 () 6) JP-7 () , 7) KDD86 () and 8) KDD-167 () . The data are the means of three experiments.

Figure 9 shows growth delay of the murine Lewis lung carcinoma after treatment of the tumor-bearing limb with fractionated radiation therapy (2, 3 or 4 Gray) daily for 5 days beginning on day 7 after tumor cell implantation alone () or along with an allosteric effector of hemoglobin (100 mg/kg) administered by intraperitoneal injection daily on days 4 through 18 after tumor cell implantation. The symbols are: 1) RSR-13 () , 2) JP-7 () , 3) KDD-86 () , 4) RSR-4 () , 5) RSR-46 () , 6) GSJ-61 () , and 7) KDD-167 () . The data are the means of three experiments.

#### Detailed Description of the Invention

The present method is directed to the use of fibric acid derivatives for treating subjects having neoplastic cells. In one embodiment these compounds can be used to inhibit neoplastic growth. In a second embodiment those compounds can be used to inhibit metastasis.

I have found that administering these compounds to subjects having solid tumors, for example Lewis lung carcinoma or MB-49 bladder carcinoma or lymphomas such as leukemia will slow down (i.e. inhibit) the growth rate of

the tumor and will also decrease the number of metastases in such subjects. Preferred targets include malignances of the breast, colon, lung, prostate and brain. Another preferred group of targets are leukemia. Preferably, the  
5 subject is a mammal, more preferably the subject is a human.

The fibric acid derivatives preferably used in the present method are those described at pages 8-15, *supra*.

These allosteric effectors were very effective in  
10 reducing the number of lung metastases in these animals.

The administration of the fibric acid derivatives can also increase the anticancer efficacy of a number of antineoplastic agents. For example, RSR-13 administration markedly augmented the anticancer efficacy  
15 of both radiation therapy and chemotherapy especially in its activity against systemic disease. Additionally, KDD-86, GSJ-61, KDD-167, RSR-46 and RSR-4 showed some toxicity toward bone marrow CFU-GM. For instance, RSR-13, RSR-4 and JP-7 were effective as additions to  
20 fractionated radiation therapy in the Lewis lung carcinoma.

The cytotoxicity of radiation toward normally oxygenated EMT-6 cells at low doses of radiation could be increased by administration by the compounds described  
25 herein; such administration could also increase the



cytotoxicity of radiation toward hypoxic cells at higher doses of radiation. Moreover exposure of malignant cells such as EMT-6 cells to the allosteric effectors increased the cytotoxicity of 4-hydroperoxy cyclo-phosphamide (4-HC), allosteric effectors could also increase the cytotoxicity of chemotherapeutics such as thiotepa and carboplatin. However, a more limited number of compounds, e.g., RSR-4 and RSR-46 increased the cytotoxicity of melphalan. RSR-13 was a highly effective modifier of chemotherapy.

The administration of the compounds disclosed herein such as RSR-13 can be cytotoxic to carcinoma cells, for example, EMT-6 murine mammary carcinoma cells. With a number of the derivatives, however, the preferred dose should be at least 500 mg/kg. Up to the dose of 500 mg/kg, a number of the allosteric effectors were not very cytotoxic toward EMT-6 tumor cells from tumors. However, the compounds did delay tumor growth. Thus, these compounds can be used to inhibit tumor growth. For example, that data establishes that RSR-13 when administered to a subject having a Lewis lung carcinoma prior to each fraction of a fractionated radiation therapy regiment delayed the growth of the tumor.

Furthermore, the administration of these compounds by the method of the present invention resulted in

decreased number of metastases, e.g. lung metastases, in tumor-bearing animals.

The number of metastases is further reduced by extended periods of administration. For example, whereas radiation or many modes of chemotherapy are administered in relatively discrete amounts spread over many months, one would administer the present compounds relatively continuously. They can be administered numerous times a week over many months. The compounds would be administered at periodic intervals of at least twice per week, more preferably at least three times per week. The periodic interval would be determined by the half-life of the compound used.

The compounds would be administered for at least two weeks, more preferably at least four weeks, still more preferably at least four weeks, still more preferably for at least six months. Most preferably the compound would be administered until the tumor is no longer at risk for shedding cells. Since these compounds are significantly less toxic than many other cytotoxic agents, such prolonged administration is possible.

These compounds can be prepared by means known in the art. See for example, WO 92/20335 and U.S. Patent No. 5,250,701. For example, 2-[4- (((aryl)amino)-carbonyl)-methyl)phenoxy]-2-methyl propionic compounds

can be made by appropriate modification of the following general procedure.

For example, 5.2 g (34 mmol) of 4-hydroxyphenyl-acetic acid (HPAA) is heated to reflux with an excess of  
5 thionyl chloride ( $\text{SOCl}_2$ ) for 1/2 hour. The reaction mixture is then cooled and excess  $\text{SOCl}_2$  is removed under vacuum. The residue is reacted for 2 hours with 6.3 g (68mmol) of for example aniline in 50 ml of refluxing xylene. The reaction mixture is then cooled, washed with  
10 dilute HCl, water and brine and extracted with aqueous 2N NaOH. The combined alkali layer is washed with ether, cooled and acidified to provide 7 g of solid N-phenyl-4-hydroxybenzyl amide ( $\text{C}_{14}\text{H}_{12}\text{NO}_2$ ) as an intermediate product (90% yield), mp  $138^\circ\text{C}$ . The intermediate product is  
15 recrystallized from a 1:2 mixture of acetone and petroleum ether and a 1.13g(5 mmol) portion is O-alkylated for 12 hours using the procedure of Example 1 with 20 ml acetone, 2.75 g NaOH and 1.25 ml  $\text{CHCl}_3$ . The final product is 2-[4-(((phenyl)amino)carbonyl)  
20 methyl)phenoxy]-2-methyl propionic acid ( $\text{C}_{18}\text{H}_{19}\text{NO}_4$ ), 1.2 g (76% yield), mp  $198^\circ\text{C}$ .

The above procedure can be used to make other compounds coming within this group. For example, RSR-13 can be made by repeating the above procedure using 3.26  
25 (21 mmol) of the HPAA, 5.3 ml (42 mmol) of 3,5-dimethyl

aniline rather than aniline, and 25 ml of refluxing xylene. In this case the intermediate product is N-(3,5-dimethylphenyl)-4-hydroxy benzylamide. 1.27 g (5 mmol) of the intermediate is used to produce 2-[4-(((3,5-dimethylphenyl) amino)carbonyl)methyl)phenoxy]-2-methyl propionic acid ( $C_{20}H_{23}NO_4$ ), 1.15 g (68% yield) mp 85C. Alternatively, the procedure outlined in the German Patent Application 2, 432, 560, which is herein incorporated by reference, can be followed to produce the compound.

RSR-13 is a member of the fibric acid class of molecules which is known to have hypolipidemic activity by increased triglyceride-rich lipoprotein catabolism through increased lipoprotein lipase activity (Grundy and Vega, 1987).

Preferably, treatment with the present method is initiated at an early point after detection of the malignancy. For example, in the lung colony assay where a large challenge of tumor cells were placed in circulation, early treatment with a compound such as RSR-13, before the tumor cells implanted in the lungs, was clearly most beneficial.

Preferably, the use of the compounds described herein are administered in conjunction with at least one second chemotherapeutic agent. Typically, the joint

administration enhances the effectiveness of each compound is inhibiting tumor growth as measured by delayed growth. Significantly, the combined administration reduces the rate of metastases in these subjects. For example, the combination of RSR-13 with a chemotherapy agent (cyclophosphamide, adiamycin, 5-fluorouracil or cis-diamminedichloroplatinum II (CDDP) resulted in 1.2 to 1.5-fold increases in the response of the primary tumor and marked decrease in lung metastases. In animals bearing the MB-49 bladder carcinoma, administration of RSR-13 resulted in increased responses to both fractionated radiation therapy and chemotherapy and marked decreases in lung metastases.

Preferred chemotherapy agents include cyclophosphamide, adiamycin, 5-fluorouracil or Cis-diamminedichloroplatinum II (CDDP).

In general, for the treatment of a neoplastic growth, for a solid tumor such as Lewis lung carcinoma, more preferably, to inhibit the metastases of such carcinomas, a suitable effective dose of one or more of the compounds disclosed herein will be in the range of 0.01 to 100,000  $\mu\text{g}$  per kilogram body weight of recipient per day, preferably in the range of 0.1 to 1,000  $\mu\text{g}$ , still more preferably in the range of 0.5  $\mu\text{g}$  to 500  $\mu\text{g}$  per kilogram body weight per day. The desired dose is

suitably administered once or several more sub-doses administered at appropriate intervals throughout the day, or other appropriate schedule. These sub-doses may be administered as unit dosage forms, for example, containing 0.01 to 100  $\mu\text{g}$ , preferably 0.5 to 100  $\mu\text{g}$ . When used in combination with a second chemotherapeutic agent, these dosages can be lower than that of either compound by itself.

Administration of the compounds used in the present invention may be by any suitable route including oral, rectal, nasal, topical (including buccal and sublingual), vaginal and parenteral (including subcutaneous, intramuscular,, intravenous and intradermal) with oral or parenteral being preferred. It will be appreciated that the preferred route may vary with, for example, the condition and age of the recipient.

While the fibric acid derivatives may be administered alone, they also may be present as part of a pharmaceutical composition. The compositions used in the present invention comprise at least one combination of compounds together with one or more acceptable carriers, e.g., liposomes, and optionally other therapeutic ingredients, including those therapeutic agents discussed supra. The carrier(s) must be "acceptable" in the sense

of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The compositions include those suitable for oral, rectal, nasal, topical (including buccal and sublingual),  
5 vaginal or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be presented in unit dosage form, e.g., tablets and sustained release capsules, and in liposomes and may be  
10 prepared by any methods well known in the art of pharmacy.

Such methods include the step of bringing into association the ingredients to be administered with the carrier which constitutes one or more accessory  
15 ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers, liposomes or finely divided solid carriers or both, and then, if necessary, shaping the product.

20 Compositions used in the present invention that are suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a  
25 suspension in an aqueous liquid or a non-aqueous liquid;



as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion; packed in liposomes; or as a bolus; etc.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients.

5 Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface-active or dispersing water. Molded tablets may  
10 be molded by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein.

15 Compositions suitable for topical administration include lozenges comprising the ingredients in a flavored base, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and  
20 mouthwashes comprising the ingredient to be administered in a suitable liquid carrier.

Compositions suitable for topical administration to the skin may be presented as ointments, creams, gels and pastes using a pharmaceutically acceptable carrier. A

suitable topical delivery system is a transdermal path containing the ingredient to be administered.

Compositions suitable for rectal administration may be presented as a suppository with a suitable base  
5 comprising, for example, cocoa butter or a salicylate.

Compositions suitable for nasal administration wherein the carrier is a solid include a coarse powder having a particle size, for example, in the range 20 to 500 microns which is administered in the manner in which  
10 snuff is taken, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid, for administration, as for example, a nasal spray or as nasal drops, include aqueous or oily  
15 solutions of the active ingredient.

Compositions suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art  
20 to be appropriate.

Compositions suitable for parental administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation  
25 isotonic with the blood of the intended recipient; and

aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and  
5 may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders,  
10 granules and tables of the kind previously described.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations used in this invention may include other agents conventional in the art having regard to the type  
15 of formulation in question, for example, those suitable for oral administration may include flavoring agents.

All documents mentioned herein are incorporated by reference.

The present invention is further illustrated by the  
20 following Examples. These Examples are provided to aid in the understanding of the invention and are not to be construed as limitations thereof.

#### MATERIALS AND METHODS

Drugs. RSR-13, . [2-4(((3,5-dimethylanilino)carbonyl)-  
25 methyl) phenoxy]-2-methyl-propionic acid, was provided by

ALLOS Therapeutics, Inc. (Denver, CO) (Figure 1). It can be prepared by the method taught infra. Cyclophosphamide and *cis*-diamminedichloroplatinum II (CDDP) were purchased from Sigma Chemical Co. (St. Louis, MO). Adriamycin and  
5 5-fluorouracil were purchased from the Dana-Farber Cancer Institute pharmacy.

Tumor Oxygen Measurements. The rat mammary adenocarcinoma 13762 is a carcinogen induced (DMBA) tumor of the female Fisher 344 rat. The tumor can metastasize  
10 to the lungs and abdominal organs. The tumor is composed of epithelial tissue in folds and acini. The tumor grows to 100 mm<sup>3</sup> in about 14 days when implanted subcutaneously in the hind legs of female rats.

Tissue oxygen measurements were made using a pO<sub>2</sub>-  
15 Histogram (Eppendorf, Inc., Hamburg, Germany). The polarographic needle microelectrode was calibrated in aqueous solutions saturated with air or 100% nitrogen. The electrode was used in tumor measurements if there was less than 0.16% variation in current measurements upon  
20 repetition of the calibration cycle. For tumor pO<sub>2</sub> measurements, the animal was anesthetized by an i.p. injection of ketaset (35 mg/kg) and xylazine (25 mg/kg) prepared in phosphate-buffered 0.9% saline. The animal was placed on a heating pad and covered with a blanket to  
25 maintain body temperature. Core temperature was measured

with a rectal thermometer. The tumor site was shaved and tumor diameters measures with calipers. A small patch of skin about 2 cm from tumor was shaved and a small incision was made allowing the reference electrode  
5 (Ag/AgCl-ECG) to be inserted subcutaneously and secured. The tumor was exposed by removing about 0.5cm<sup>2</sup> of skin over the site. The tumor capsule was perforated with a 20-gauge needle. The pO<sub>2</sub> microelectrode was positioned in the perforation.

10 The pO<sub>2</sub> measurements were made under several conditions: 1) normal air breathing, 2) carbogen (95% O<sub>2</sub>/5% CO<sub>2</sub>) breathing, 3) immediately after intravenous administration of RSR-13 (100, 150 or 200 mg/kg) with air breathing, 4) immediately after intravenous  
15 administration of RSR013 (100, 150 or 200 mg/kg) with carbogen breathing, 5) 30 minutes after intravenous administration of RSR-13 (100, 150 or 200 mg/kg) with air breathing, 6) 30 minutes after intravenous administration of RSR-13 (100, 150 or 200 mg/kg) with air breathing, and  
20 8) 60 minutes after intravenous administration of RSR-13 (100, 150 or 200 mg/kg) with carbogen breathing.

In another study, RSR-13 (200 mg/kg) was administered by continuous intravenous infusion over 60 minutes and pO<sub>2</sub> measurements were made at 0, 5, 20, 40  
25 and 60 minutes during the infusion and 30 minutes after

the completion of the infusion. Data collection through three tumor diameters accrued about 50 pO<sub>2</sub> measurements and took about 10 minutes. The pO<sub>2</sub> microelectrode was recalibrated in aqueous solutions saturated with air and 100% nitrogen after each data collection; therefore, the pO<sub>2</sub> microelectrode was recalibrated 4 times during the course of the experiment. Recalibration requires about 15 minutes (Teicher et al., 1994a; Teicher et al., 1995b; Teicher et al., 1994b; Teicher et al., 1993b; Teicher et al., 1994c).

Tumor Excision Assay. The EMT-6 murine mammary carcinoma which is an *in vivo-in vitro* tumor system was used for these experiments (Rockwell, 1977; Rockwell, 1978). The EMT-6 tumor was carried in Balb/C mice (Taconic Farms, Germantown, NY). For the experiments, 2x10<sup>6</sup> tumor cells prepared from a brei of several stock tumors were implanted intramuscularly into the legs of Balb/C mice 8- to 10-weeks old. On day 8 after tumor cell implantation when the tumors were about 150 mm<sup>3</sup> in volume, RSR-13 (100, 300 or 500 mg/kg) was administered as a single dose by intraperitoneal injection. The mice were killed 24 hours after treatment to allow for full expression of drug cytotoxicity and repair of potentially lethal damage and then soaked in 95% ethanol. The tumors were excised, and single cell suspensions were prepared (Teicher et

al., 1990; Teicher et al., 1987). The untreated tumor cell suspensions had a plating efficiency of 8% to 12%. The results are expressed as the surviving fraction  $\pm$  S.E.M. of cells from treated groups compared with untreated controls.

Bone Marrow Toxicity. Bone marrow was taken from the same animals used for the tumor excision assay. A pool of marrow from the femurs of two animals was obtained by gently flushing the marrow through a 23-gauge needle and a colony formation was carried out as described previously (Teicher et al., 1990; Teicher et al., 1987). Colonies of at least 50 cells were scored on an Acculite Colony counter (Fisher, Springfield, NJ). The results from three experiments, in which each group was measured at three cell concentrations in duplicate, were averaged and are expressed as the surviving fraction of treated groups compared with untreated controls.

Tumor Growth Delay. The Lewis lung carcinoma and the MB-49 bladder carcinoma growing in C57BL mice were chosen for tumor growth delay studies because these tumors are relatively resistant to many cancer therapies and are highly metastatic to the lungs from s.c. implants. The Lewis lung carcinoma and the MB-49 bladder carcinoma were carried in male C57BL mice (Taconic Farms). For experiments,  $2 \times 10^6$  Lewis lung tumor cells or  $2 \times 10^6$



MB-49 bladder carcinoma cells prepared from a brei of several stock tumors were implanted s.c. into the legs of male mice 8-10 weeks of age.

**Experiment 1.** Animals bearing the Lewis lung carcinoma  
5 were treated with RSR-13 (100 or 200 mg/kg) administered intraperitoneally daily on days 7 through 11 post tumor cell implantation when the tumors were about 100 mm<sup>3</sup> in volume. The RSR-13 was administered either immediately before or 30 minutes before radiation therapy. Radiation  
10 therapy was delivered in fractions of 0, 2, 3 or 4 Gray daily for 5 days locally to the tumor-bearing limb (Gamma Cell 40, Atomic Energy of Canada, Ottawa; dose rate 0.88 Gy/min).

**Experiment 2.** Animals bearing the Lewis lung carcinoma  
15 were treated with RSR-13 (50, 100 or 200 mg/kg) administered intraperitoneally daily from day 4 through day 18 post tumor cell implantation. On day 7 post tumor cell implantation, radiation therapy was initiated. The animals were treated with radiation fractions of 0, 2, 3  
20 or 4 Gray daily for 5 days locally to the tumor-bearing limb as described above.

**Experiment 3.** Animals bearing the Lewis lung carcinoma were treated with RSR-13 (100 or 200 mg/kg) administered by intraperitoneal injection on days 7 through 11 post  
25 tumor cell implantation immediately prior to

administration of the cytotoxic chemotherapy. The  
chemotherapeutic agents were: 1) cyclophosphamide (150  
mg/kg) administered by intraperitoneal injection on days  
7, 9 and 11; 2) adriamycin(1.75 mg/kg) administered by  
5 intraperitoneal injection on days 7 through 11; 3) 5-  
fluorouracil (30 mg/kg) administered by intraperitoneal  
injection on days 7 through 11; and 4) CDDP (cisplatin,  
10 mg/kg) administered as a single intraperitoneal  
injection on day 7. Other animals bearing the Lewis lung  
10 carcinoma were treated as indicated in Table 5 and Figure  
9.

**Experiment 4.** Animals bearing the MB-49 bladder  
carcinoma were treated with RSR-13 (100 or 200 mg/kg)  
administered by intraperitoneal injection on days 7  
15 through 11 post tumor cell implantation alone or along  
with a cytotoxic anticancer therapy. The cytotoxic  
therapies were: 1) fractionated radiation therapy (3  
Gray) administered locally to the tumor-bearing limb  
daily on days 7 through 11 as described above; 2)  
20 cyclophosphamide (100 mg/kg) administered by  
intraperitoneal injection on days 7, 9 and 11; or 3)  
adriamycin (1.25 mg/kg) administered by intraperitoneal  
injection on days 7 through 11.

**Additional Experiments.** Using the above described  
25 techniques animals with EMT-6 murine mammary carcinoma

cells were treated as indicated in the legends to Figures 6 through 8.

The progress of each tumor was measured thrice weekly until it reached a volume of 500 mm<sup>3</sup>. Tumor growth delays was calculated as the days taken by each individual tumor to reach 500 mm<sup>3</sup> compared with the untreated controls. Each treatment group had 6 animals and each experiment was repeated 3 times (n=18). Days of tumor growth delay are the mean  $\pm$  SE for the treatment group compared with the control group. Tumor growth delay is the difference in days for treated vs. control tumors to reach 500 mm<sup>3</sup> (Teicher et al., 1995 a). Control tumors reached 500 mm<sup>3</sup> as follows: (1) Lewis lung carcinoma, 14. $\pm$  0.9 days; and (2) MB-49 bladder carcinoma, 14.1  $\pm$  1.1, post-s.c. implantation (Teicher et al., 1995a).

Lung metastasis were examined on day 20. Untreated control animals died from lung metastases on days 21-25. The numbers of external lung metastases were counted from 2 animals per group and scored as  $\geq$  3 mm or less in diameter. Metastases  $\geq$  3 mm in diameter were counted as large (vascularized) (Teicher et al., 1995a).

Lung Colony Assay. Animals were injected with 10<sup>4</sup> Lewis lung carcinoma cell intravenously on day 0. The animals were untreated or were treated with RSR-13 (100 or 200

mg/kg) administered by intraperitoneal injection on days -1 through 3, days 0 through 4, days 1 through 5, days 3 through 7 or days 7 through 11. Lungs were collected on day 20. The numbers of external lung metastases were counted and scored for size as described above (Teicher et al., 1988).

## RESULTS

The structure of RSR-13 and the related fabric acid derivatives, bezafibrate and clofibric acid, are shown in Figure 1. RSR-13 has been shown to bind specifically in the central water cavity of deoxyhemoglobin, thereby shifting the allosteric equilibrium of hemoglobin for oxygen such that oxygen is more readily released from the hemoglobin molecule (Abraham et al., 1995; Abraham et al., 1992). Rats bearing the 13762 mammary carcinoma were injection intravenously with RSR-13 (100, 150 or 200 mg/kg) and the oxygen content of the tumor was determined using an Eppendorf  $pO_2$  histogram to determine the effect of RSR-13 administration on tumor oxygenation, (Table 1).

20

Under air breathing immediately after RSR-13 administration there was no significant change in the median  $pO_2$  of the tumor or in the hypoxic faction, defined as the percent of  $pO_2$  readings < 5 mmHg of the tumor. Thirty minutes after RSR-13 administration, the

25

median  $pO_2$  of the tumor increased significantly to about 17 mmHg from about 6 mmHg in the controls. The hypoxic fraction of the tumor was significantly decreased with the RSR-13 dose of 150 mg/kg. By 60 minutes after RSR-13 administration the median  $pO_2$  in the tumor was returning to control levels as was the tumor hypoxic fraction. Breathing carbogen (95% oxygen/5% carbon dioxide) along with RSR-13 administration markedly increased tumor oxygenation. Immediately after injection of RSR-13 with carbogen breathing the median  $pO_2$  was about 30 mmHg; by 30 minutes after RSR-13 administration the median  $pO_2$  was about 46 mmHg and by 60 minutes after RSR-13 administration the median  $pO_2$  was down to about 26 mmHg. The administration of RSR-13 along with carbogen breathing altered the oxygenation profile of the 13762 tumor so that the oxygen distribution was no longer skewed to low levels of oxygen but was spread over a broad range of oxygen levels (Figure 2).

To determine whether RSR-13 administered by infusion could stably decrease hypoxia in the 13762 tumor, RSR-13 (200 mg/kg) was infused over 60 minutes intravenously into rats and tumor  $pO_2$  measurements were made at 5 time points during the infusion and 30 minutes after completion of the infusion (Figure 3). The hypoxic fraction of the tumor decreased from 49% to 25% by 40

minutes into the infusion period and then started to rise so that by 30 minutes after completion of the infusion of RSR-13 it had returned to the pretreatment level. The median  $pO_2$  in the tumor rose over the initial 5 minutes of the RSR-13 infusion then rose more slowly to about 14 mmHg at 40 minutes into the infusion. By 30 minutes after completion of the RSR-13 infusion the tumor median  $pO_2$  had returned to baseline.

The EMT-6 tumor *in vivo/in vitro system* was used to assess the cytotoxicity of RSR-13 and other fibric acid derivatives toward tumor cells and bone marrow CFU-GM after treatment of tumor bearing mice with the drug (Figures 4 and 6-9). RSR-13 in single doses up to 500 mg/kg was administered by intraperitoneal injection. Twenty-four hours after RSR-13 administration the tumors were excised and known numbers of tumor cells were plated in cell culture for colony formation. Fifty percent of the tumor cells were killed by a dose of 340 mg/kg of RSR-13. The highest dose of RSR-13 (500 mg/kg) killed about 80% of the tumor cells. RSR-13 was not toxic toward bone marrow CFU-GM. Initial tumor growth delay studies were carried out using the Lewis lung carcinomas. RSR-13 at a dosage of 100 or 200 mg/kg was administered alone or in conjunction with radiation therapy. Two experimental endpoints were assessed: 1) growth delay of

the subcutaneous tumor and 2) number and size of lung metastases on day 20 (Table 2).

The other compounds tested were relatively non-cytotoxic toward EMT-6 mammary carcinoma cells in culture with  $IC_{50}$ 's between 100  $\mu$ M and > 500  $\mu$ M upon 24 hour exposure. Exposure of hypoxic EMT-6 cells to allosteric effector molecules (100  $\mu$ M) for 1 hour prior to and during x-ray delivery resulted in dose modifying factors between 2 and 3. Simultaneous exposure of EMT-6 cells to an allosteric effector AE (100  $\mu$ M) and a concentration range of thiotepa, carboplatin, melphalan or 4-HC resulted in a potentiation of cytotoxicity of 2-3 logs increase in cell killing.

Administration of RSR-13 daily for five days produced a 2.3 day and 2.7 day growth delay of the subcutaneous tumor at the 100 mg/kg and 200 mg/kg doses. Fractionated radiation therapy produced increasing growth delay of the Lewis lung carcinoma with increasing radiation dose. The addition of RSR-13 (100 mg/kg) prior to each radiation fraction resulted in increased tumor growth delay and a radiation dose modifying factor of 1.25. Administration of the higher dose of RSR-13 prior to each radiation fraction further increased the tumor growth delay and produced a radiation dose modifying factor of 1.63. Delaying radiation delivery



until 30 minutes after RSR-13 administration increased the tumor response to radiation at both doses of RSR-13 so that radiation dose modifying factors of 1.55 and 1.66 resulted with RSR-13 (100 mg/kg) and RSR-13 (200 mg/kg),  
5 respectively.

The Lewis lung carcinoma metastasizes avidly to the lungs from the subcutaneously implanted primary tumor. On day 20 post tumor cell implantation, the control animals had a mean of 23 lung metastases, 62% of which  
10 were large enough to be angiogenic (Table 2). Administration of RSR-13 (100 or 200 mg/kg) decreased both the number of lung metastasis, 62% of which were large decreased both the number of lung metastasis to about 13 and the percent of large lung metastases to  
15 about 40%. Radiation therapy locally to the tumor-bearing limb did not significantly alter the number or size of lung metastasis on day 20. The combination of RSR-13 administration and radiation therapy, especially with the higher doses of radiation, further decreased the  
20 number of lung metastases to about 8 and the percent of large lung metastases to about 28%.

Extending the RSR-13 treatment time to days 4 through 18 was assessed in combination with fractionated radiation therapy on days 7 through 11 post Lewis lung  
25 carcinoma implantation (Table 3). RSR-13 (50, 100 or 200



mg/kg) administered alone produced a tumor growth delay between 2.4 and 3.0 days. The radiation dose modifying factors resulting from these treatment combinations were 1.28, 1.50 and 1.66 at RSR-13 doses of 50, 100 and 200  
5 mg/kg, respectively. Treatment with RSR-13 alone decreased the number and size of lung metastases to about the same levels as seen with the five days RSR-13 regimen. In combination with fractionated radiation, however, the longer RSR-13 treatment resulted in  
10 decreases in the number of lung metastases to 4 - 8 and percentages of large lung metastases of 8% to 25% at the 4 Gray radiation dose.

Administration of other allosteric effectors (See Fig. 9 and Table 5) (50 or 100 mg/kg) daily on days 4-18  
15 following tumor implant resulted in little or no growth delay of SC Lewis lung carcinoma but decreased lung metastases to 30-50% of the number in control animals.

Radiation sensitization and chemosensitization in vivo varied with substitution of the base molecule such  
20 that molecules with dimethyl-substitution on the phenyl ring were effective sensitizers while ethoxy-, chloro- and cyclophentyl-substitution resulted in some loss of activity. Variation in the fibric acid portion of the molecule seemed less important.

Most systemically administered anticancer chemotherapy produces host toxicity. For initial treatment combination studies of RSR-13 with anticancer drugs, treatment agents which are widely used and represent major classes of antitumor drugs were chosen. RSR-13 was administered simultaneously with each anticancer drug on days 7 through 11 post implantation of the Lewis lung carcinoma (Table 4). Cyclophosphamide (150 mg/kg) produced a 17.4-day tumor growth delay in the Lewis lung carcinoma. The addition of RSR-13 (100 mg/kg) resulted in a 1.3 fold increase in tumor growth delay compared with cyclophosphamide alone. The addition of RSR-13 (200 mg/kg) to cyclophosphamide treatment resulted in early deaths of the animals. Adriamycin (1.75 mg/kg) produced 6.8 days of growth delay in the Lewis lung carcinoma. The addition of RSR-13 (100 or 200 mg/kg) to treatment with adriamycin modestly increased the tumor growth delay observed. 5-Fluorouracil produced 3.9 days of tumor growth delay in the Lewis lung carcinoma. The addition of RSR-13 to treatment with 5-fluorouracil resulted in 1.2-fold and 1.4-fold increases in tumor growth delay at the RSR-13 doses of 100 mg/kg and 200 mg/kg, respectively.

Treatment with CDDP resulted in 4.5 days of growth delay in the Lewis lung carcinoma. The addition of RSR-

13 to treatment with CDDP resulted in 1.3-fold and 1.5-fold increases in tumor growth delay at the RSR-13 doses of 100 mg/kg and 200 mg/kg, respectively, compared to treatment with CDDP alone.

5           As was observed with radiation therapy, there was a marked reduction in the number of lung metastases on day 20 in treatment regiments combining RSR-13 and a chemotherapeutic agent compared with chemotherapy alone. Each of the cytotoxic anticancer drugs decreased the  
10       number of lung metastases on day 20. The addition of RSR-13 (100 mg/kg) to cyclophosphamide treatment decreased the number of lung metastases to 1.5 compared to 12 after cyclophosphamide treatment only. Similarly, the number of lung metastases observed after addition of  
15       RSR-13 (200 mg/kg) to treatment with adriamycin or 5-fluorouracil was 1.5 compared to 13 and 6 after treatment with each of the anticancer drugs alone, respectively. The addition of RSR-13 to treatment with CDDP did not alter the number of lung metastases observed compared  
20       with treatment with CDDP alone.

          The antimetastatic effects of a compound such as RSR-13 were unexpected. To confirm that the positive therapeutic effects of RSR-13 would generalize to other metastatic tumors, the MB-49 bladder carcinoma was  
25       selected as a second tumor for study. Treatment with

RSR-13 (100 or 200 mg/kg) produced 2 to 3 days of growth delay in the MB-49 bladder carcinoma (Table 6). These RSR-13 treatments also decreased the number of lung metastases in these animals from 26 to 16-17.

5           Fractionated radiation therapy (3 Gray) delivered on days 7 through 11 post tumor cell implantation produced 4.5 days of tumor growth delay. Adding administration of RSR-13 to the fractionated radiation therapy regimen resulted in a 1.3 fold and 1.4 fold increase in tumor  
10 growth delay at RSR-13 doses of 100 mg/kg and 200 mg/kg, respectively, compared to the tumor growth delay obtained with radiation therapy only. Cyclophosphamide (100 mg/kg) produced a tumor growth delay of 11.4 days. The addition of RSR-13 (100 or 200 mg/kg) to treatment with  
15 cyclophosphamide resulted in a 1.4-fold increase in tumor growth delay compared with cyclophosphamide alone. Adriamycin (1.25 mg/kg) produced a 4.3 day growth delay of the MB-49 bladder carcinoma. The addition of RSR-13 at doses of 100 or 200 mg/kg to treatment with adriamycin  
20 resulted in increases in tumor growth delay of 1.3-fold or 1.6 fold, respectively. Each of the cytotoxic anticancer therapies decreased the number of lung metastases on day 20 compared with the untreated controls.

The addition of RSR-13 to each cytotoxic therapy further decreased the number of lung metastases on day 20. The number of lung metastases after local fractionated radiation therapy decreased to 8 from 14 when administered along with RSR-13 (200 mg/kg). The number of lung metastases after cyclophosphamide treatment decreased from 4.5 to 0.5 when RSR-13 (200 mg/kg) was added to the regimen. The number of lung metastases after adriamycin treatment decreased from 7 to 4 when RSR-13 (200 mg/kg) was added to the regimen.

Metastasis is a complex process. To begin to define the point at which RSR-13 might act in the metastatic process, the ability of RSR-13 to inhibit Lewis lung carcinoma cell colony formation was assessed (Figure 5). Lewis lung carcinoma cells ( $10^4$ ) were injected intravenously in the tail of C57BL mice on day 0 and lung colonies were counted on day 20. RSR-13 (100 or 200 mg/kg) was administered by intraperitoneal injection daily for 5 days beginning one day prior to tumor cell injection, the day of tumor cell injection or 1, 3 or 7 days after tumor cell injection. In this assay, the 100 mg/kg dose of RSR-13 was more effective than the 200 mg/kg dose. RSR-13 was most effective at inhibiting lung colony formation by Lewis lung carcinoma cells when drug administration was initiated prior to tumor cell

injection, followed by drug treatment initiation at the time of tumor cell injection. RSR-13 continued to lose effectiveness as the time between tumor cell injection and drug administration lengthened. When tumor cell injection preceded RSR-13 treatment initiation by 3 or 7 days, there was no significant difference between the number of lung colonies in the RSR-13 treated animals and the untreated control animals.

In another embodiment, these compounds reduce hypoxia. Hypoxia has been shown to protect malignant cells in culture and in solid tumors from the cytotoxic actions of radiation therapy and of many chemotherapeutic agents (Teicher, 1995). RSR-13, a non-toxic small molecule, acts on the hemoglobin within red blood cells altering its conformation so that oxygen is released more readily from the hemoglobin into the tissues (Abraham et al., 1995; Abraham et al. 1992; Khandelwal et al., 1993; Wei et al., 1993). Thereby, RSR-13 administration provides a period of time during which tumor oxygenation is increased and during which radiation therapy or chemotherapy can be administered. In the current study, the time period of increased tumor oxygenation after bolus administration of RSR-13 appears to be at 15-30 minutes after administration of the drug and to have a duration of 20 to 30 minutes. Breathing a high oxygen

content atmosphere enhances the tumor oxygenating activity of RSR-13.

RSR-4, JP-7, KDD-86, RSR-46, GSJ-61 and KDD-167 also resulted in reduction of tumor metastasizes and delays in  
5 tumor growth as well as increasing cytotoxicity of an antineoplastic agent such as radiation.

It is evident that those skilled in the art given the benefit of the foregoing disclosure may make numerous other uses and modifications thereof and departures from  
10 the specific embodiments described herein without departing from the inventive concepts, and the present invention is to be limited solely by the scope and spirit of the appended claims.

TABLE 1. Oxygen-related parameters (percent and median  $pO_2$  of  $pO_2$  readings  $\leq 5$  mmHg) for the rat 13762 mammary carcinoma under normal air breathing and carbogen (95% oxygen/5% carbon dioxide) breathing conditions alone or after intravenous administration of RSR-13. The data are based on 500-3000 measured  $pO_2$  values.

Treatment Group	Oxygenation Condition (% $\leq 5$ mmHg/Median, mmHg)	
	air	carbogen
Controls	49%/5.8	36%/19.6
Immediately after:		
RSR-13 (100 mg/kg)	62%/2.1	34%/24.2
RSR-13 (150 mg/kg)	47%/8.6	15%/35.7
RSR-13 (200 mg/kg)	62%/2.2	41%/30.9
30 min. after:		
RSR-13 (100 mg/kg)	43%/13.8	34%/38.7
RSR-13 (150 mg/kg)	15%/21.5	0%/67
RSR-13 (200 mg/kg)	43%/17.0	29%/32
60 min. after:		
RSR-13 (100 mg/kg)	53%/9.9	40%/27.1
RSR-13 (150 mg/kg)	55%/2.1	43%/26.5
RSR-13 (200 mg/kg)	61%/6.9	32%/23.6



TABLE 2. Growth delay of the Lewis lung carcinoma and number and percent large lung metastases on day 20 after short-term treatment with RSR-13 and fractionated radiation therapy.

Treatment Group	Tumor Growth Delay, Days			Number of Lung Metastases (% Large)		
	Alone	RSR-13 5 x 100 mg/kg	5 x 200 mg/kg	Alone	5 x 100 mg/kg	5 x 200 mg/kg
—	—	2.3 ± 0.3	2.7 ± 0.4	2.3 (62)	14 (39)	12 (41)
RSR-13 in Immediate Sequence with X-rays						
5 x 2 Gray	3.2 ± 0.3	3.3 ± 0.3	4.7 ± 0.5	20 (55)	12 (39)	10 (40)
5 x 3 Gray	4.3 ± 0.4	6.2 ± 0.7	8.0 ± 0.8	19 (52)	10 (38)	8 (40)
5 x 4 Gray	6.2 ± 0.6	7.2 ± 0.8	9.7 ± 0.9	17 (53)	9 (32)	7 (21)
RSR-13 Wait 30 min. then X-rays						
5 x 2 Gray	3.2 ± 0.3	4.7 ± 0.5	5.0 ± 0.5	20 (55)	13 (42)	10 (42)
5 x 3 Gray	4.3 ± 0.4	6.5 ± 0.6	7.4 ± 0.8	19 (52)	11 (40)	7 (39)
5 x 4 Gray	6.2 ± 0.6	9.1 ± 0.9	10.3 ± 0.9	17 (53)	9 (39)	7 (23)

TABLE 3. Growth delay of the Lewis lung carcinoma and number and percent of large lung metastases on day 20 after long-term treatment with RSR-13 and fractionated radiation therapy.

Treatment Group	Tumor Growth Delay, Days	Number of Lung Metastases (% Large)
Controls	—	23 (62)
5 x 2 Gray	$3.2 \pm 0.3$	20 (55)
5 x 3 Gray	$4.3 \pm 0.4$	19 (52)
5 x 4 Gray	$6.2 \pm 0.6$	17 (53)
RSR-13 (50 mg/kg) d 4-18	$2.4 \pm 0.3$	13 (33)
above + 5x2 Gray	$3.9 \pm 0.4$	10.5 (43)
above + 5x3 Gray	$5.7 \pm 0.6$	9.5 (37)
above + 5x4 Gray	$7.6 \pm 0.8$	8 (25)
RSR-13 (100 mg/kg) d 4-18	$2.9 \pm 0.3$	10 (34)
above + 5x2 Gray	$4.7 \pm 0.5$	8 (44)
above + 5x3 Gray	$6.5 \pm 0.8$	6 (30)
above + 5x4 Gray	$8.7 \pm 0.9$	5 (18)
RSR-13 (200 mg/kg) d 4-18	$3.0 \pm 0.3$	12 (42)
above + 5x2 Gray	$4.9 \pm 0.6$	9 (33)
above + 5x3 Gray	$6.9 \pm 0.8$	7 (21)
above + 5x4 Gray	$10.5 \pm 1.0$	4 (8)

TABLE 4. Growth delay of the Lewis lung carcinoma and number and percent large lung metastases on day 20 after treatment with RSR-13 and chemotherapeutic agents. <sup>a</sup>

Treatment Group	Tumor Growth Delay, Days			Number of Lung Metastases (% Large)		
	Alone	RSR-13		Alone	RSR-13	
		5 x 100 mg/kg	5 x 200 mg/kg		5 x 100 mg/kg	5 x 200 mg/kg
	—	2.6 ± 0.4	3.0 ± 0.4	24 (60)	14 (50)	8.5 (41)
Cyclophosphamide (3 x 150 mg/kg)	17.4 ± 1.3	22.4 ± 1.8	-Toxic -	12 (53)	1.5 (66)	—
Adriamycin (5 x 1.75 mg/kg)	6.8 ± 0.5	7.1 ± 0.5	7.4 ± 0.7	13 (50)	3.5 (29)	1.5 (66)
5-Fluorouracil (5 x 30 mg/kg)	3.9 ± 0.4	4.8 ± 0.5	5.3 ± 0.6	6 (50)	5.5 (52)	1.5 (100)
CDDP (10 mg/kg)	4.5 ± 0.5	5.7 ± 0.7	6.8 ± 0.9	13.5 (52)	14 (61)	12 (54)

<sup>a</sup> All treatments were by intraperitoneal injection on days 7 through 11. RSR-13 was administered immediately prior to the anticancer drug. Cyclophosphamide was administered on days 7, 9 and 11 and CDDP was administered as a single dose on day 7. Adriamycin and 5-fluorouracil were administered on days 7 through 11.

Table 5

Growth delay of the Lewis lung carcinoma and number and percent large lung metastases on day 20 after long-term treatment with various allosteric effectors of hemoglobin and fractionated radiation therapy.

TREATMENT GROUP	TUMOR GROWTH DELAY, DAYS	NUMBER OF LUNG METASTASES (% LARGE)
Controls	—	25 (60)
5 x 2 Gray <sup>a</sup>	3.2 ± 0.3	21 (56)
5 x 3 Gray	4.3 ± 0.4	20 (52)
5 x 4 Gray	6.2 ± 0.6	18 (52)
RSR-13 (100 mg/kg) d4-18	2.9 ± 0.3	10 (34)
above + 5 x 2 Gray	4.7 ± 0.5	8 (44)
above + 5 x 3 Gray	6.5 ± 0.8	6 (30)
above + 5 x 4 Gray	8.7 ± 0.9	5 (16)
JP-7 (100 mg/kg) d4-18	2.2 ± 0.3	8 (25)
above + 5 x 2 Gray	4.5 ± 0.4	4 (31)
above + 5 x 3 Gray	6.1 ± 0.5	3 (25)
above + 5 x 4 Gray	8.9 ± 0.8	2 (5 (40)
KDD-86 (100 mg/kg) d4-18	2.6 ± 0.3	14 (35)
above + 5 x 2 Gray	3.9 ± 0.3	13 (39)
above + 5 x 3 Gray	4.3 ± 0.4	12 (43)
above + 5 x 4 Gray	5.6 ± 0.5	9 (29)
[RSR-4 (50 mg/kg) d4-18]	3.0 ± 0.3	12 (46)
above + 5 x 2 Gray	6.3 ± 0.5	11 (33)
above + 5 x 3 Gray	7.0 ± 0.5	10 (35)
above + 5 x 4 Gray	8.6 ± 0.7	3 (50)
RSR-46 (100 mg/kg) d4-18	1.4 ± 0.3	13 (35)
above + 5 x 2 Gray	4.0 ± 0.3	13 (35)
above + 5 x 3 Gray	4.1 ± 0.4	12 (35)
above + 5 x 4 Gray	4.9 ± 0.4	8 (40)
GSJ-61 (100 mg/kg) d4-18	2.0 ± 0.3	15 (25)
above + 5 x 2 Gray	5.4 ± 0.5	12 (35)
above + 5 x 3 Gray	5.8 ± 0.6	10 (35)
above + 5 x 4 Gray	6.7 ± 0.6	7 (35)
KDD-167 (100 mg/kg) d4-18	1.7 ± 0.3	17 (44)
above + 5 x 2 Gray	2.1 ± 0.3	16 (34)
above + 5 x 3 Gray	5.9 ± 0.5	16 (34)
above + 5 x 4 Gray	6.7 ± 0.6	14 (32)

<sup>a</sup>Radiation was delivered locally to the tumor-bearing limb on days 7 through 11. Drugs were administered by intraperitoneal injection.

Table 6

Growth delay of the MB-49 bladder carcinoma and number and percent large lung metastases on day 20 after short-term treatment with RSR-13 and cytotoxic anticancer therapies.

Treatment Group	Tumor Growth Delay, Days	Number of Lung Metastases (% Large)
Controls	—	26 (36)
RSR-13 (5x100 mg/kg)	2.1 ± 0.3	17 (23)
RSR-13 (5x200 mg/kg)	3.0 ± 0.3	16 (28)
5x3 Gray	4.5 ± 0.3	14 (32)
above + RSR-13 (5x100 mg/kg)	5.7 ± 0.4	9.5 (32)
above + RSR-13 (5x200 mg/kg)	6.2 ± 0.5	8 (38)
Cyclophosphamide (3x100 mg/kg)	11.4 ± 1.0	4.5 (18)
above + RSR-13 (5x100 mg/kg)	15.8 ± 1.3	1 (0)
above + RSR-13 (5x200 mg/kg)	16.2 ± 1.4	0.5 (0)
Adriamycin (5x1.25 mg/kg)	4.3 ± 0.3	7 (14)
above + RSR-13 (5x100 mg/kg)	5.6 ± 0.4	5 (0)
above + RSR-13 (5x200 mg/kg)	6.9 ± 0.6	4 (0)

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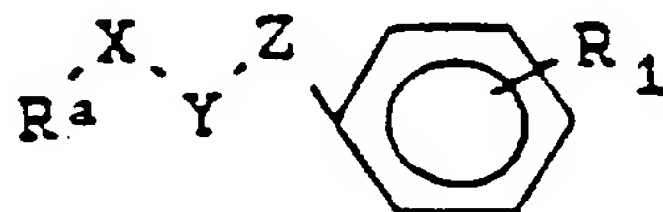
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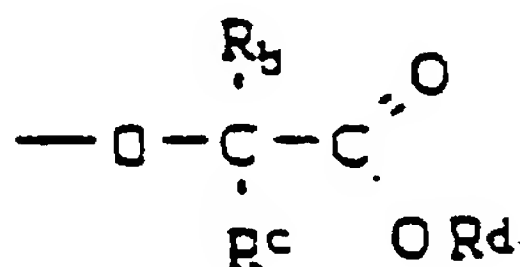
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## I CLAIM:

1. A method of treating a solid tumor or lymphoma in a mammal comprising administering an effective amount of a formula:



where  $\text{R}_1$  is a substituted or unsubstituted aromatic compound, or a substituted or unsubstituted alkyl ring compound, or a substituted or unsubstituted phthalimide compound where X is a carboxyl, Y is a nitrogen and  $\text{R}_2$  completes the phthalimide compound by being bonded to both X and Y, and Z are  $\text{CH}_2$ , NH, CO, O or N, where said X, Y, and Z moieties are each different from one another, and where  $\text{R}_1$  has the formula:



where  $R_1$  can be connected to any position on the phenyl ring, and

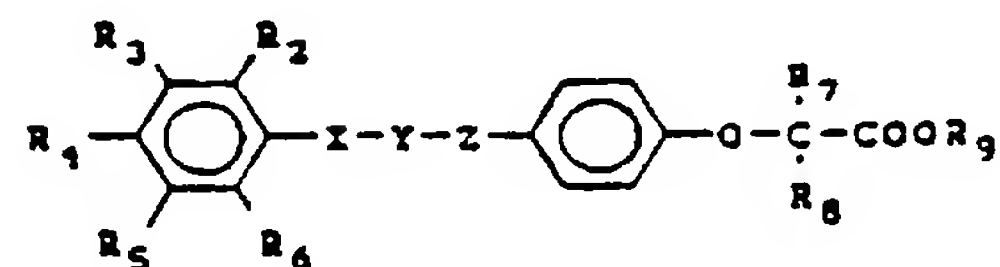
where  $R_b$  and  $R_c$  are hydrogen, halogen, methyl, or ethyl groups and these moieties may be the same or different, or alkyl moieties as part of an aliphatic ring connection  $R_b$  and  $R_c$ , and

where  $R_d$  is a hydrogen, halogen,  $C_{1-3}$  lower alkyl, or a salt cation.

2. The method of claim 1, wherein said compound is administered prior to other treatment against the solid tumor or lymphoma.
3. The method of claim 1, which further comprises determining whether said mammal is at risk for the formation of metastases, and administering to said mammal at risk, said compound at periodic intervals of at least twice per week for at least four weeks to inhibit the formation of metastases.
4. The method of claim 1 or 3, wherein said compound is administered in conjunction with at least one additional treatment regiment.
5. The method of claim 4, wherein said additional treatment regiment is radiation or a chemotherapeutic agent.



6. A method of treating a solid tumor or lymphoma in a mammal comprising administering an effective amount of a compound of the formula:



wherein X and Z may each be CH<sub>2</sub>, CO, NH or O, and Y may be CO or NH, where X, Y, and Z moieties are each different from one another;

and wherein R<sub>2-6</sub> are either hydrogen, halogen, or a substituted or unsubstituted C<sub>1-3</sub> alkyl group, or a C<sub>1-3</sub> ether or ester, and these moieties may be the same or different, or alkyl moieties of an aromatic or aliphatic ring incorporating two of the R<sub>2-6</sub> sites,

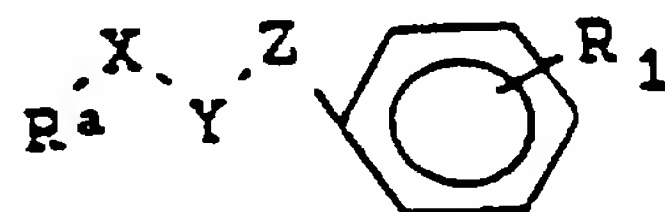
and where R<sub>7,8</sub> are hydrogen, halogen, methyl or ethyl groups and these moieties may be the same or different, or alkyl moieties as part of an aliphatic ring connecting R<sub>7</sub> and R<sub>8</sub>,

and where R<sub>9</sub> is a hydrogen, halogen, substituted or unsubstituted C<sub>1-3</sub> lower alkyl, or a salt cation.

7. The method of claim 6 wherein the compound is 2-[4-(((3-5-dimethyl phenyl)amino)carbonyl)methyl]phenoxy]-2-methyl propionic acid.

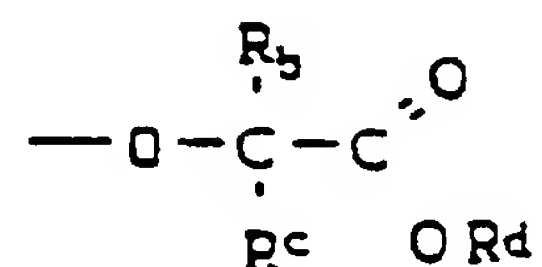
8. The method of claim 6, wherein said compound is administered prior to other treatment against the solid tumor or lymphoma.

9. The method of claim 6, which further comprises determining whether said mammal is at risk for the formation of metastases, and administering to said mammal at risk, said compound at periodic intervals of at least twice per week for at least four weeks to inhibit the formation of metastases.
10. The method of claim 6, 7 or 9, wherein said compound is administered in conjunction with at least one additional treatment regiment.
11. The method of claim 10, wherein said additional treatment regiment is radiation or a chemotherapeutic agent.
12. The use of an allosteric effector in enhancing the cytotoxicity of an antineoplastic agent.
13. The use of an allosteric effector of the formula



where  $R_1$  is a substituted or unsubstituted aromatic compound, or a substituted or unsubstituted alkyl ring compound, or a substituted or unsubstituted phthalimide compound where X is a carboxyl, Y is a nitrogen and  $R_2$  completes the phthalimide compound by being bonded to both X and Y, and Z are  $CH_2$ , NH, CO, O or N, where said

X, Y, and Z moieties are each different from one another, and where  $R_1$  has the formula:



where  $R_1$  can be connected to any position on the phenyl ring, and

where  $R_b$  and  $R_c$  are hydrogen, halogen, methyl, or ethyl groups and these moieties may be the same or different, or alkyl moieties as part of an aliphatic ring connection  $R_b$  and  $R_c$ , and

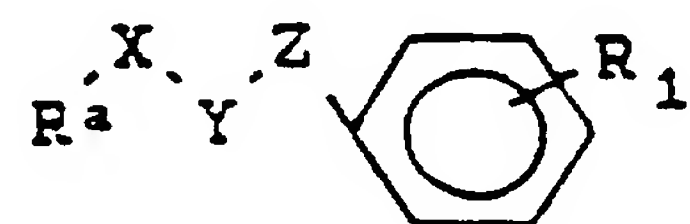
where  $R_d$  is a hydrogen, halogen,  $C_{1-3}$ , lower alkyl, or a salt cation.

for enhancing the cytotoxicity of an antineoplastic agent.

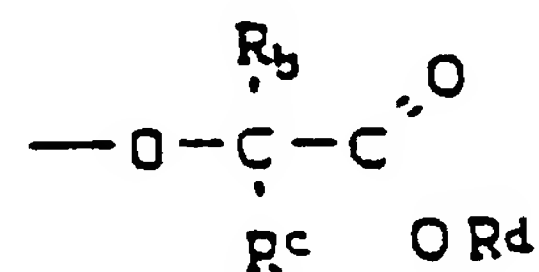
14. The use of the allosteric effector of claims 12 or 13, wherein the antineoplastic agent is radiation.

15. The use of an allosteric effector to inhibit tumor metastases.

16. The use of an allosteric effector of the formula



where  $R_1$  is a substituted or unsubstituted aromatic compound, or a substituted or unsubstituted alkyl ring compound, or a substituted or unsubstituted phthalimide compound where X is a carboxyl, Y is a nitrogen and  $R_2$  completes the phthalimide compound by being bonded to both X and Y, and Z are  $\text{CH}_2$ , NH, CO, O or N, where said X, Y, and Z moieties are each different from one another, and where  $R_1$  has the formula:



where  $R_1$  can be connected to any position on the phenyl ring, and

where  $R_b$  and  $R_c$  are hydrogen, halogen, methyl, or ethyl groups and these moieties may be the same or different, or alkyl moieties as part of an aliphatic ring connection  $R_b$  and  $R_c$ , and

where  $R_d$  is a hydrogen, halogen,  $\text{C}_{1-3}$  lower alkyl, or a salt cation.

to inhibit tumor metastases.

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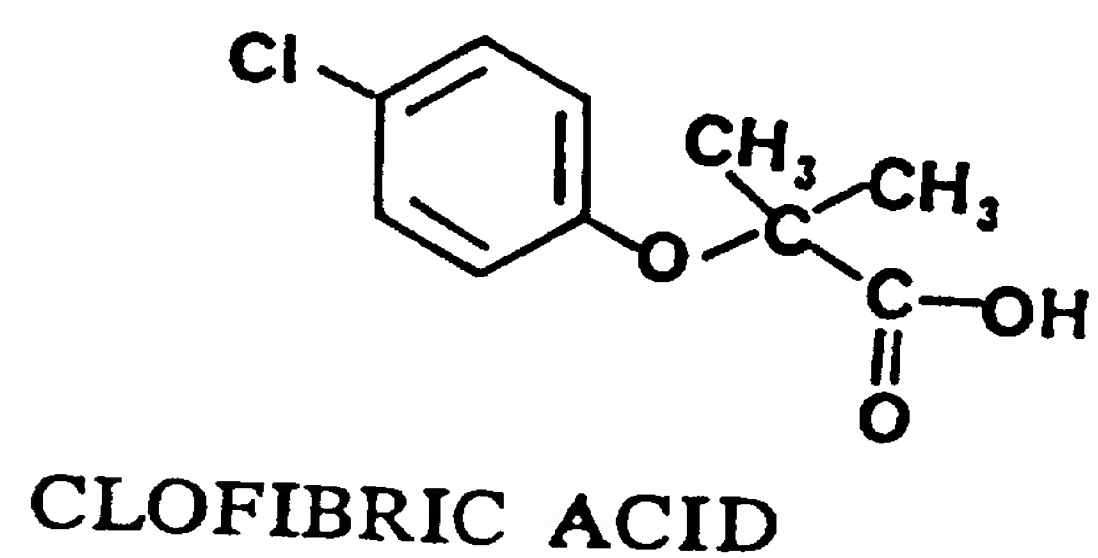
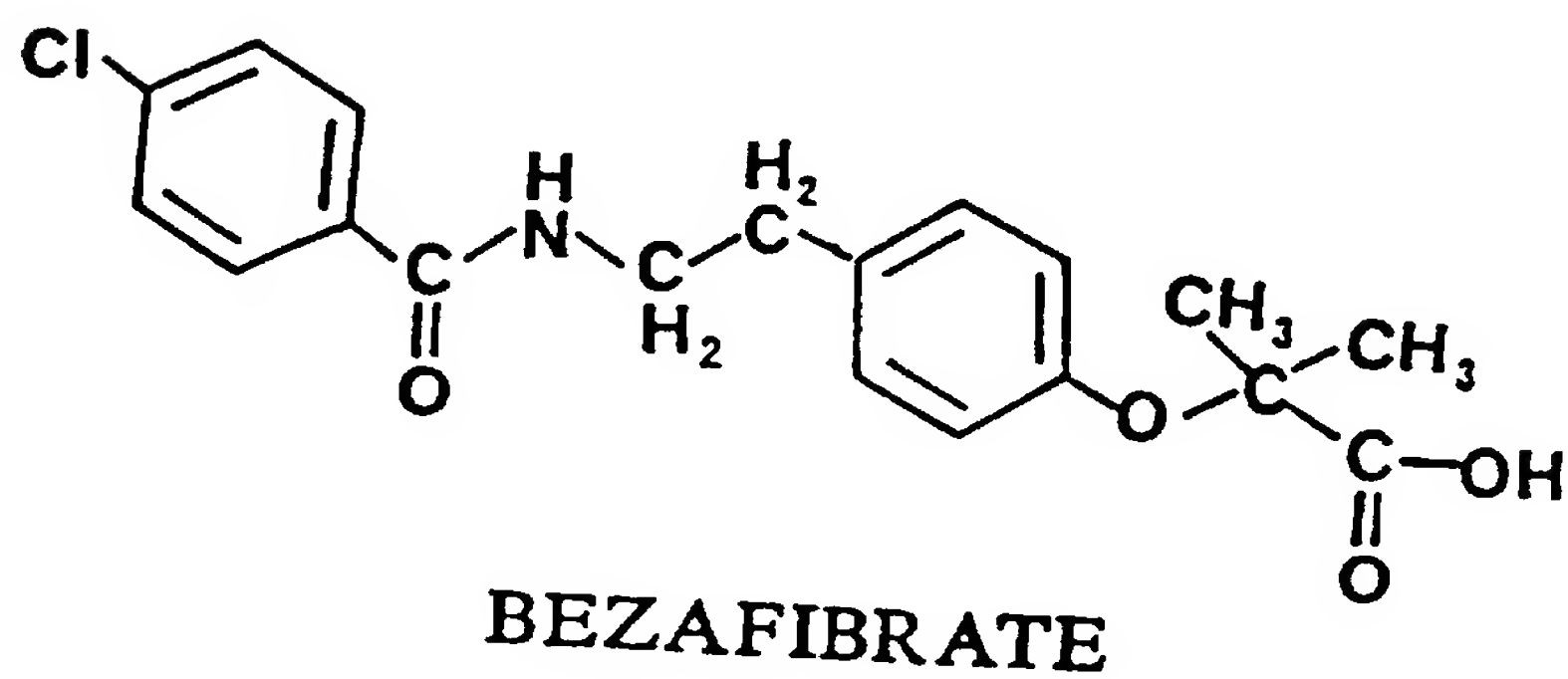
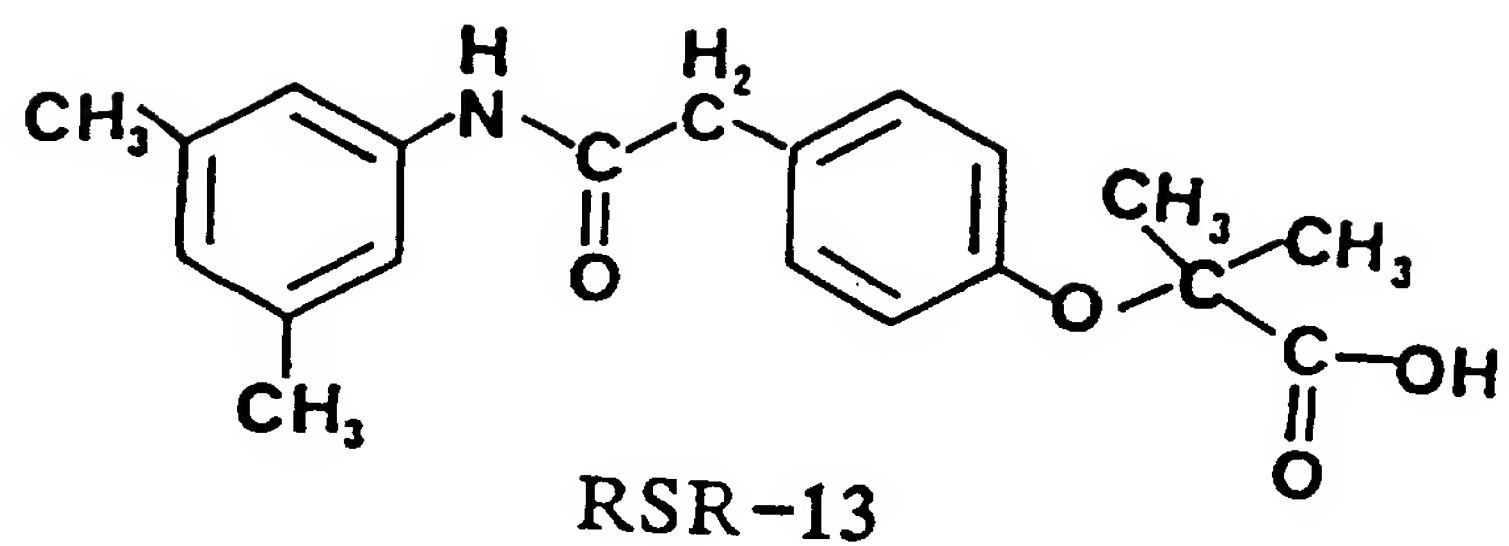


FIG. 1

**% <5mmHg**  
**Median, mmHg**

**CONTROLS**

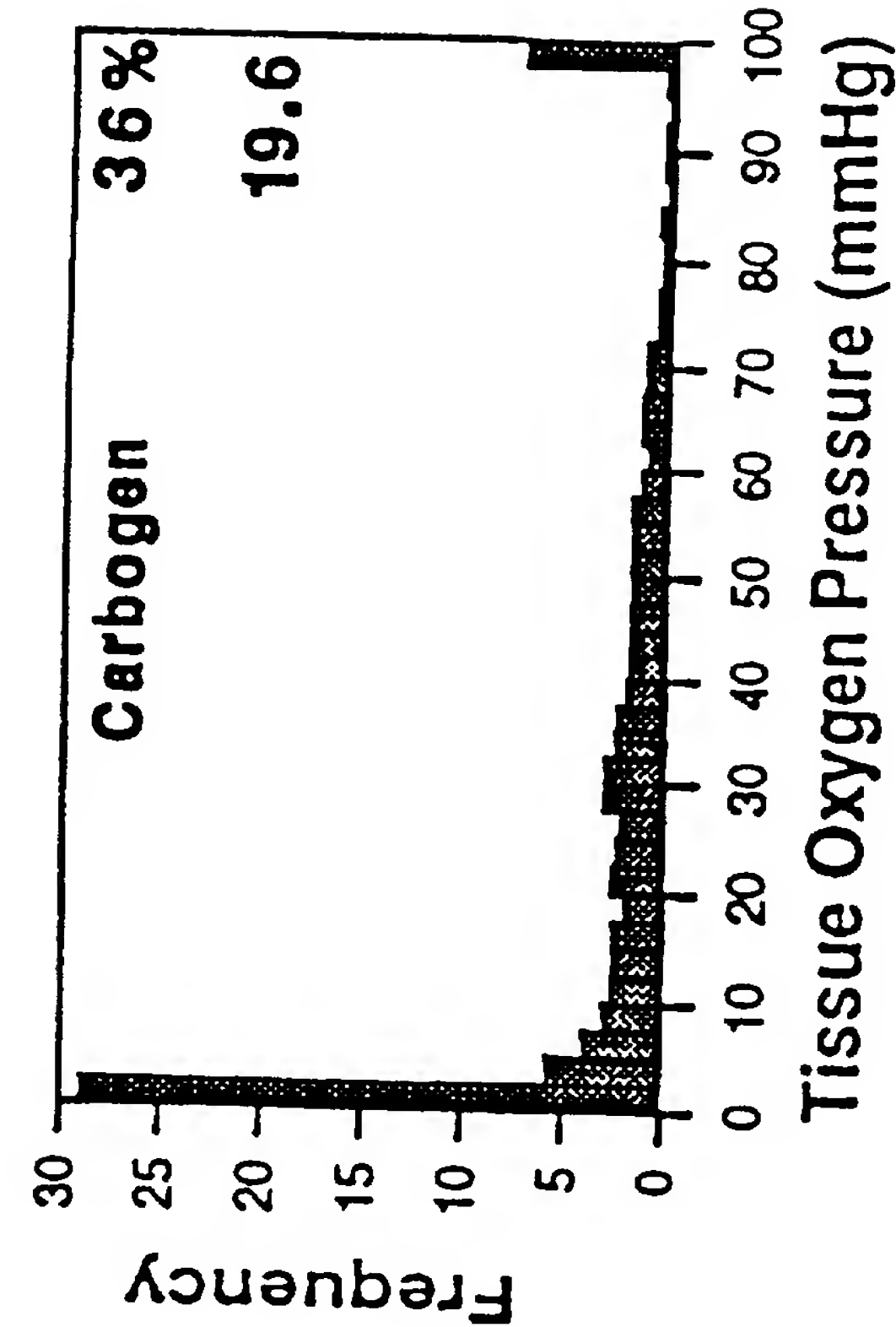


FIG. 2B

**% <5mmHg**  
**Median, mmHg**

**CONTROLS**

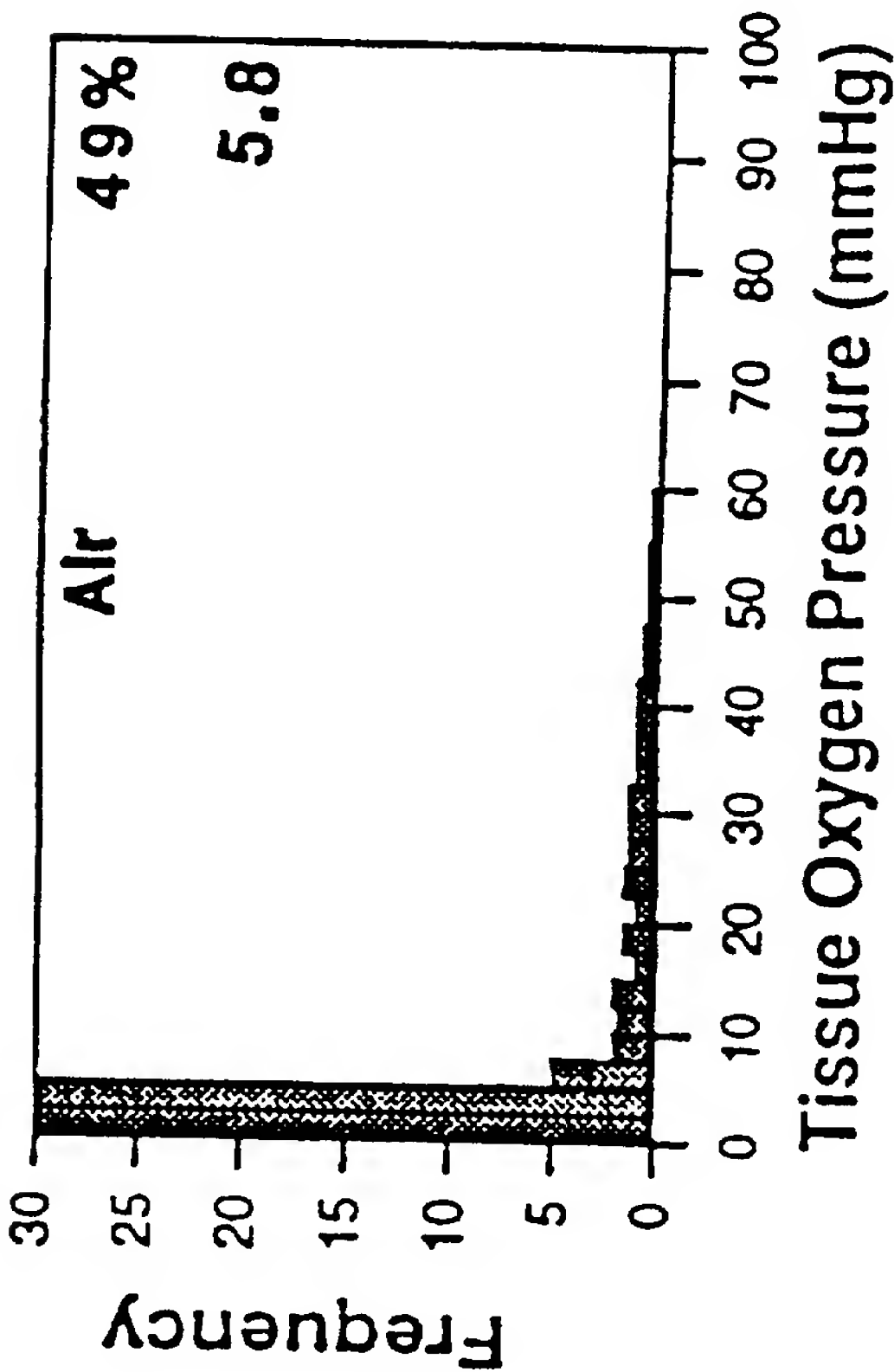


FIG. 2A

\_\_\_\_\_  
% <5mmHg  
Median, mmHg  
\_\_\_\_\_  
CONTROLS

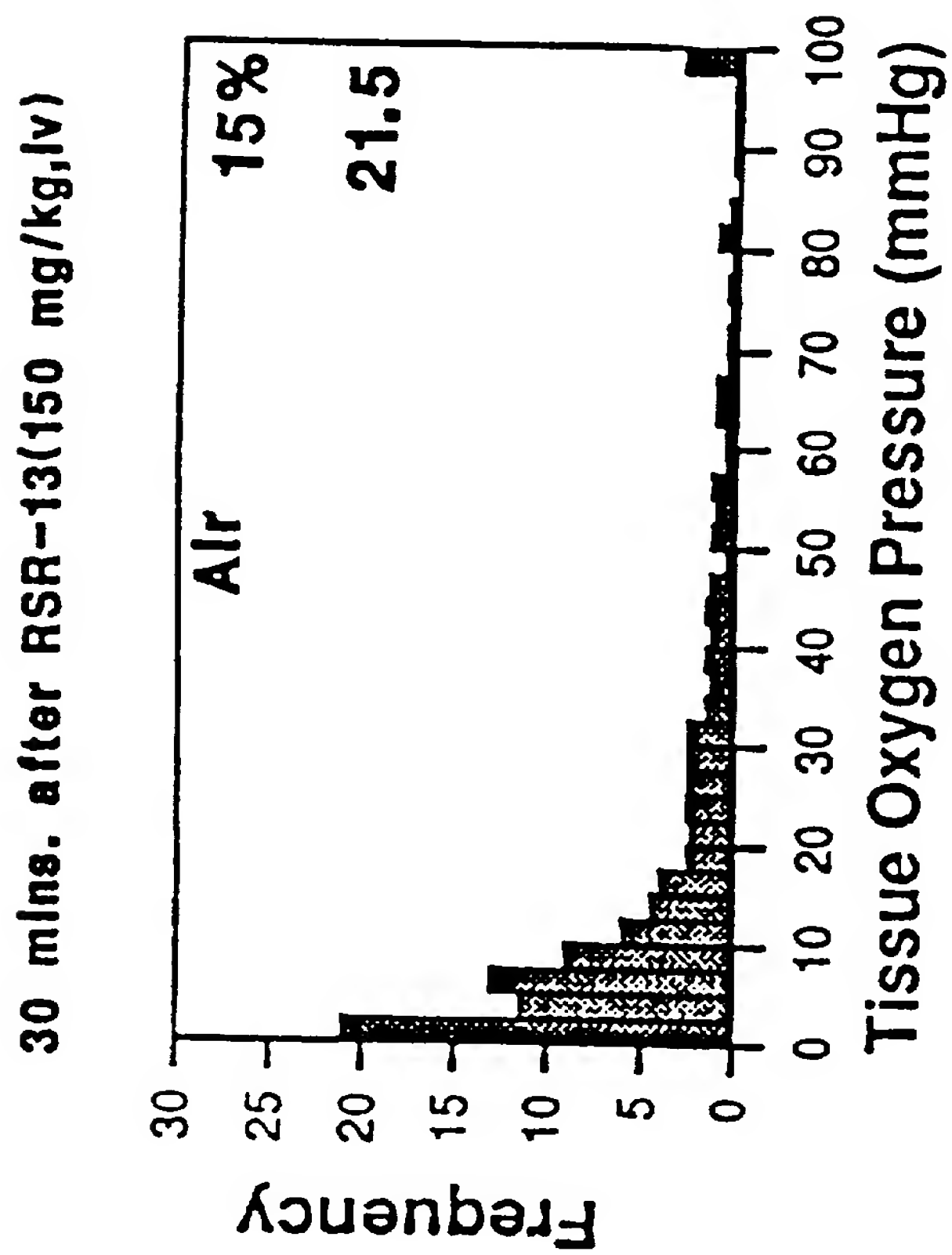


FIG. 2C

\_\_\_\_\_  
% <5mmHg  
Median, mmHg  
\_\_\_\_\_  
CONTROLS

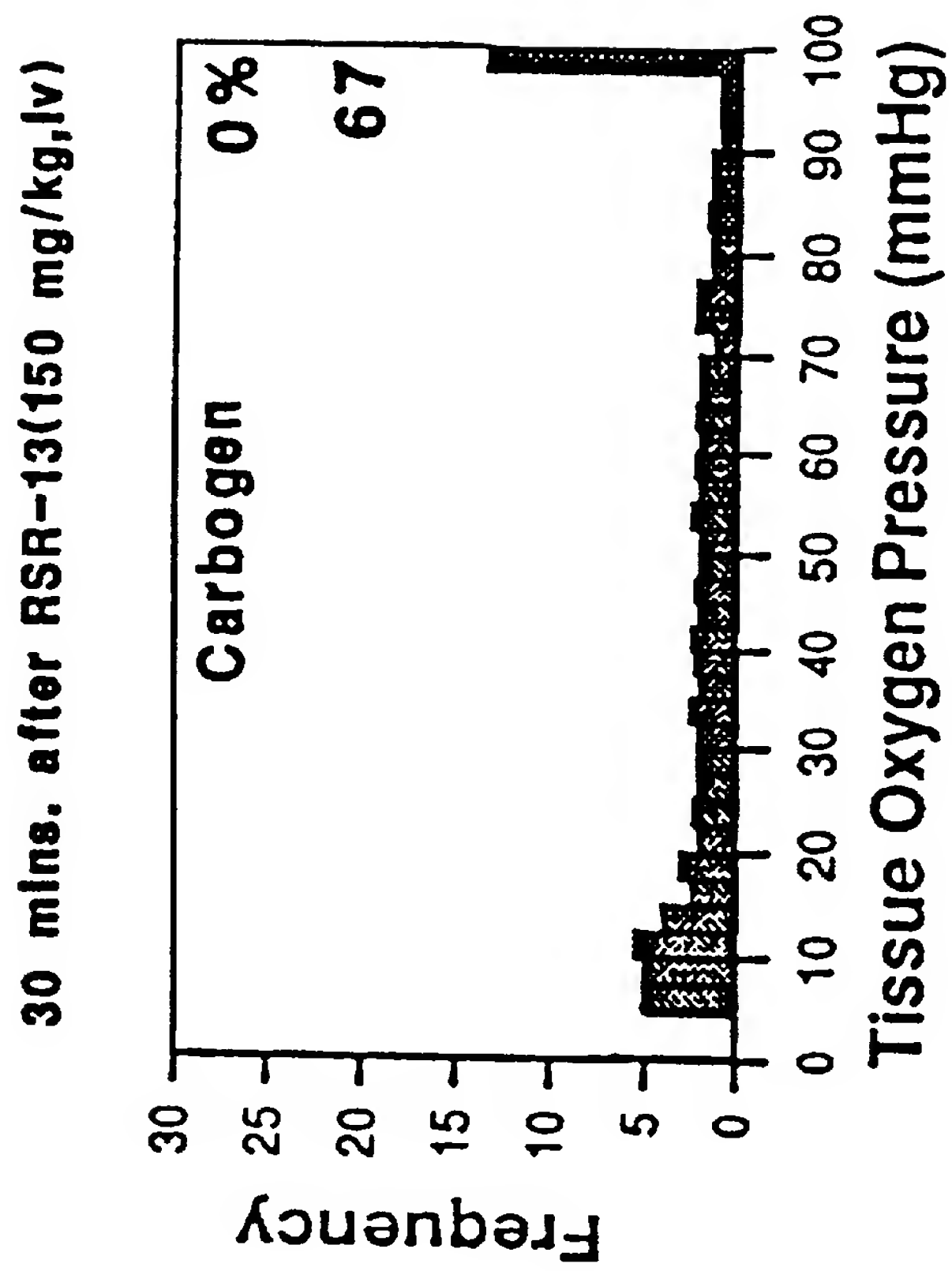


FIG. 2D



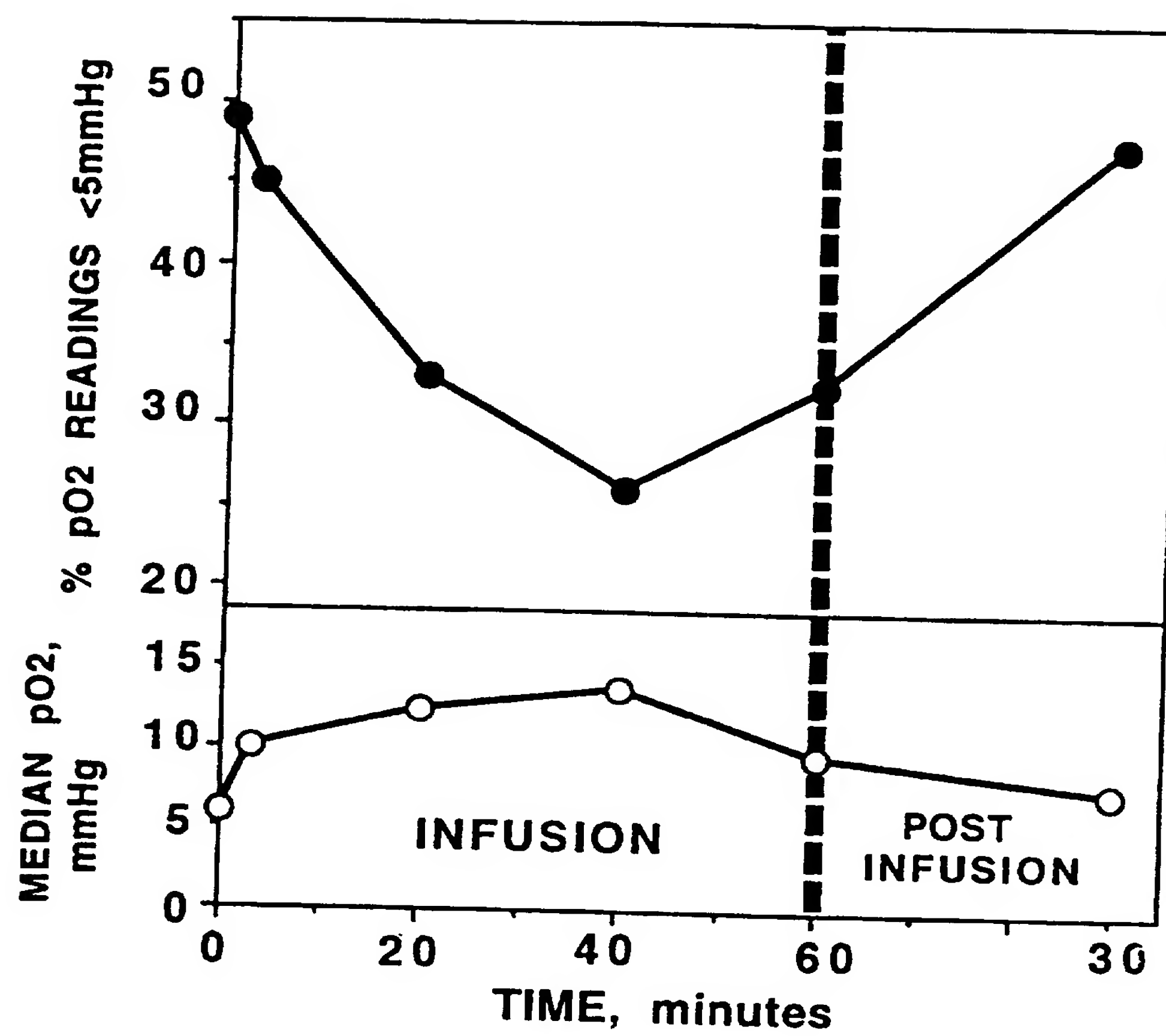


FIG. 3

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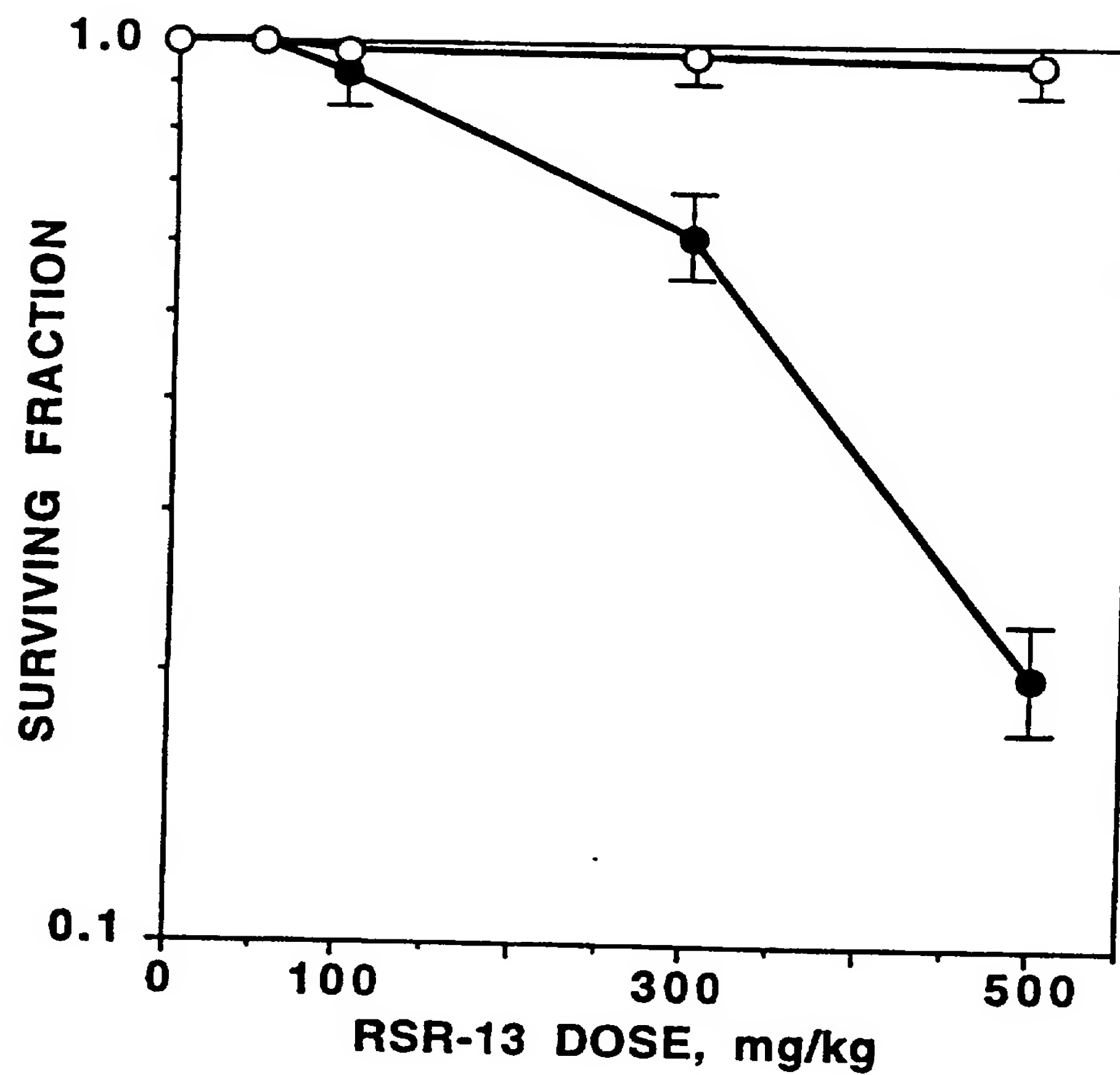


FIG. 4

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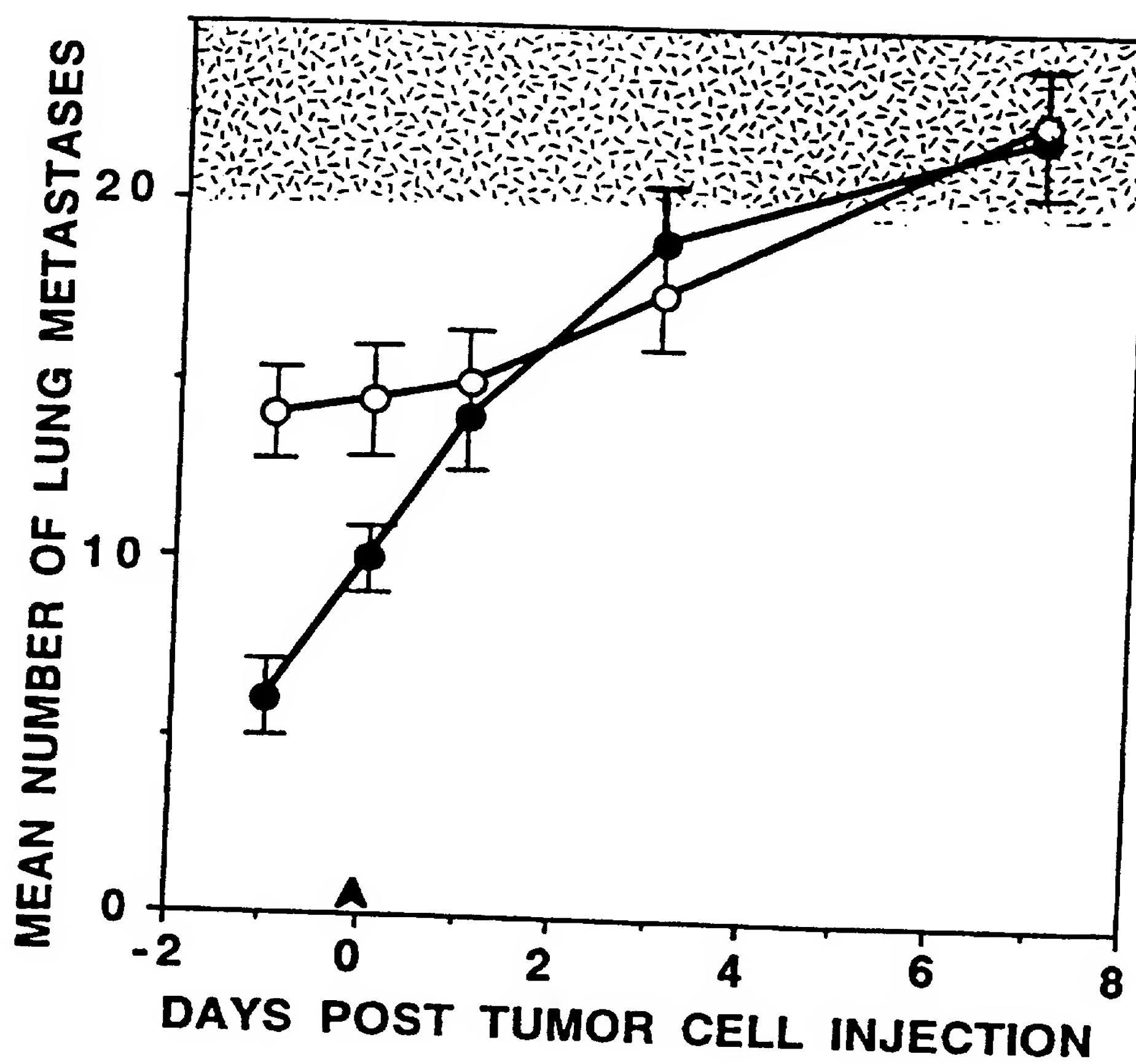


FIG. 5

SUBSTITUTE SHEET (RULE 26)

FIG.6A

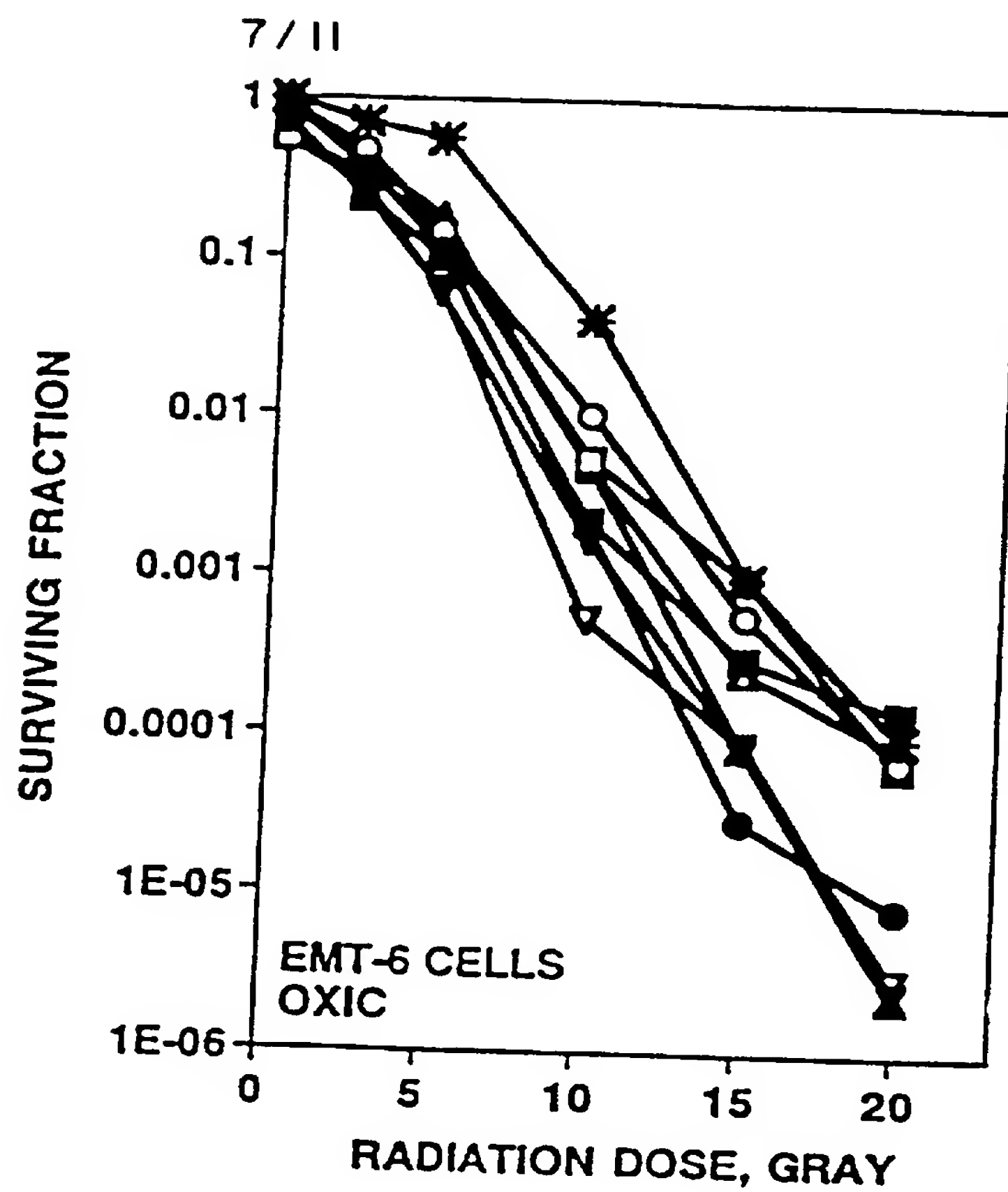


FIG.6B

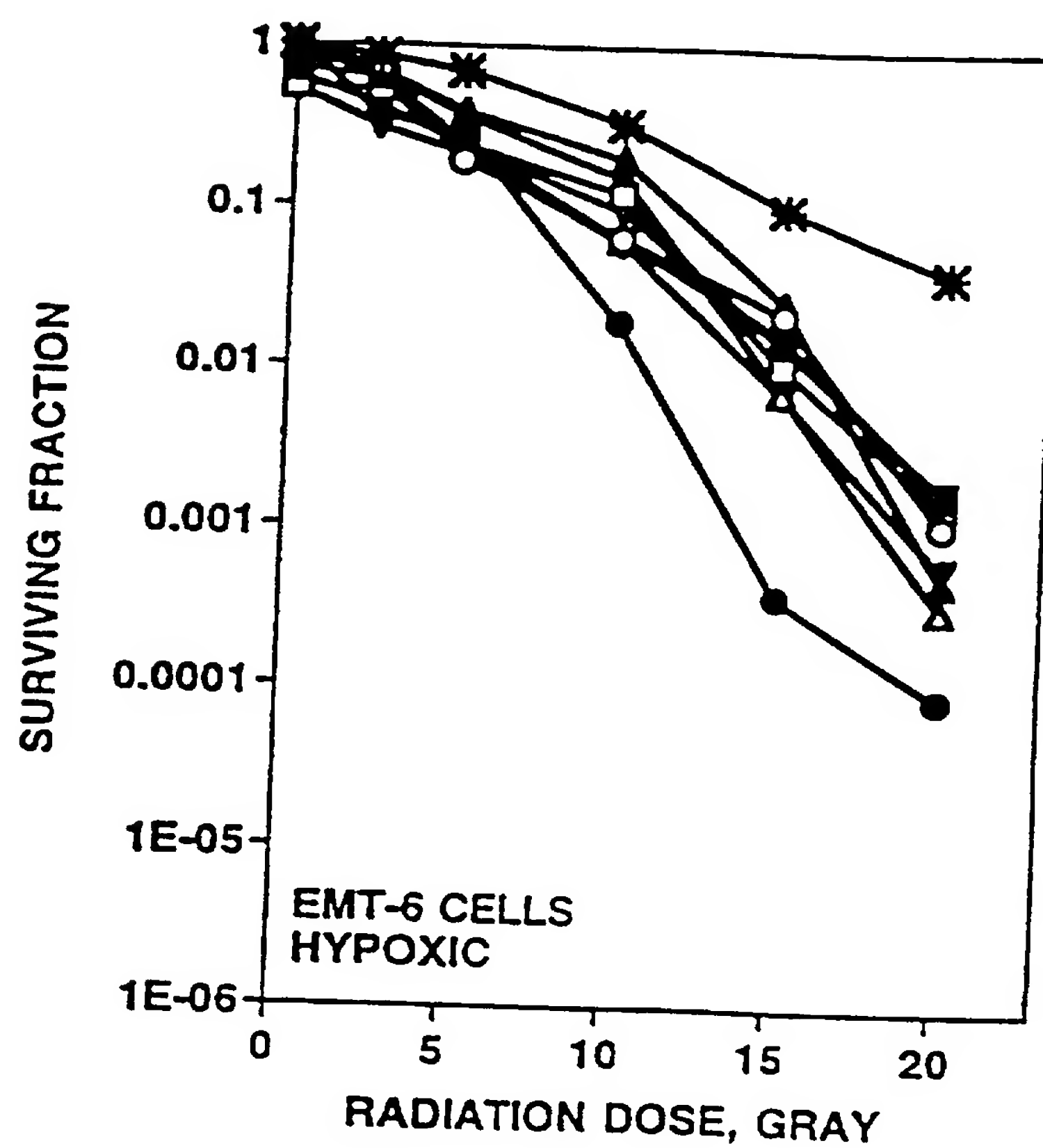


FIG. 7A

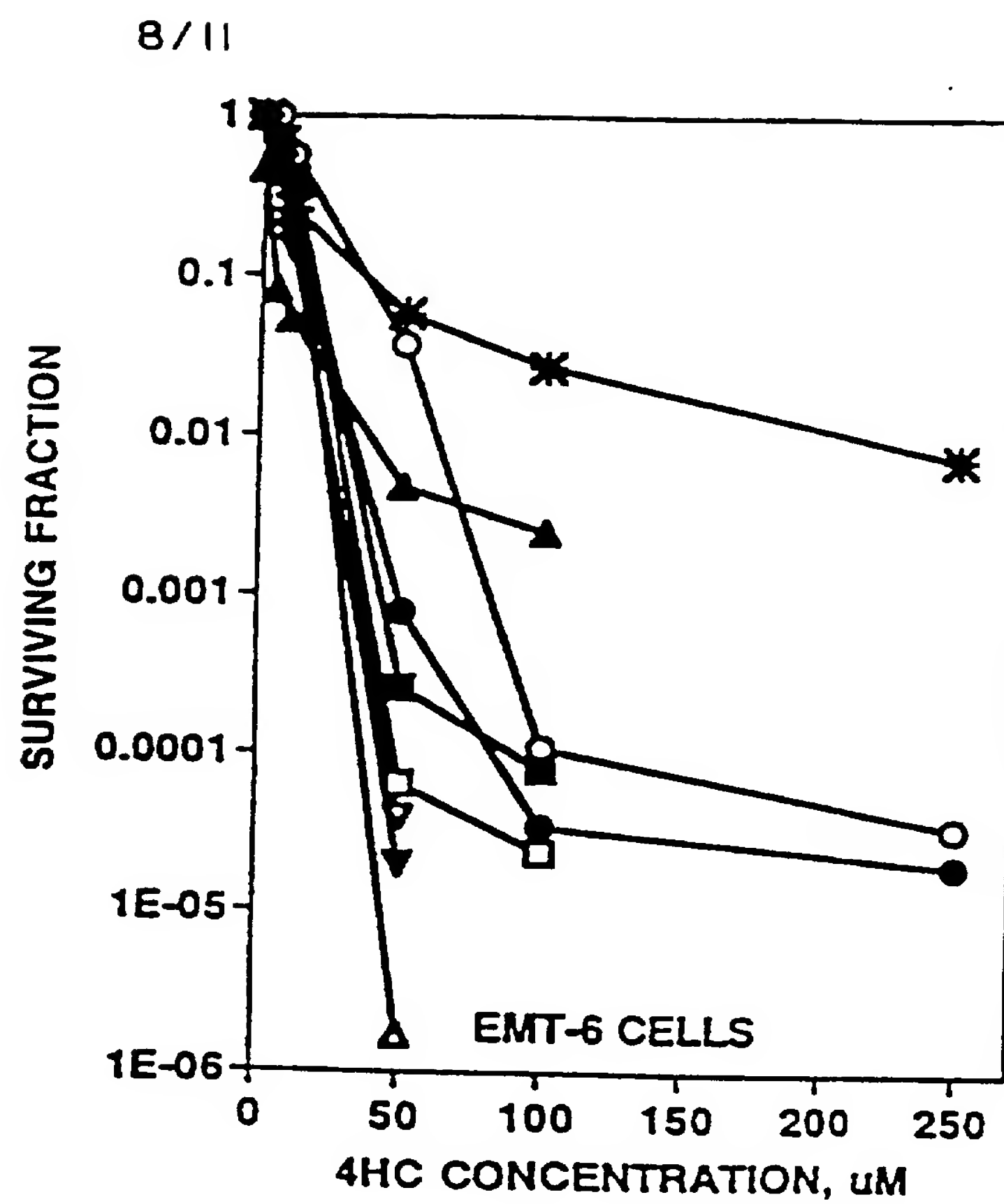
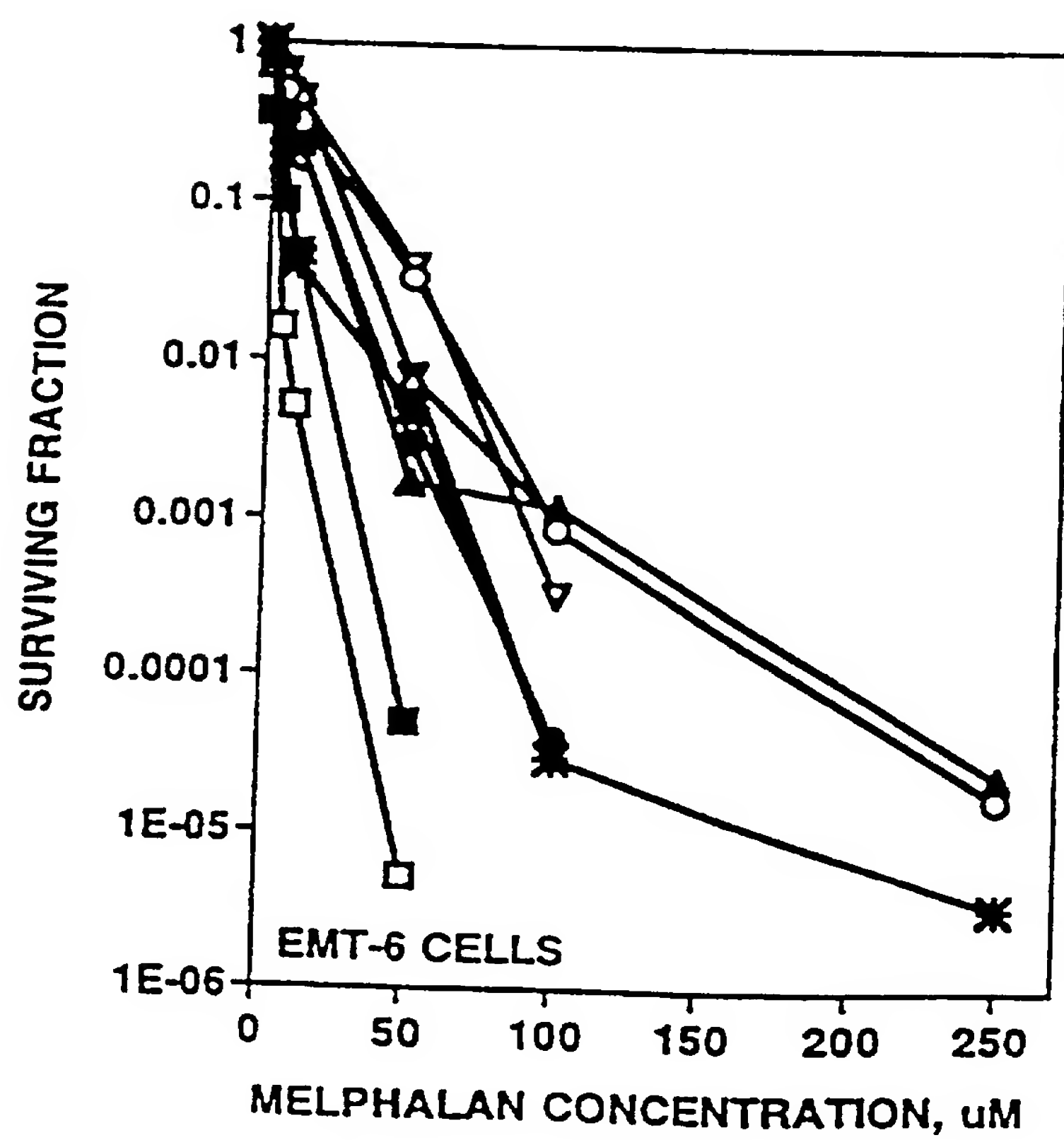


FIG. 7B



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FIG. 7C

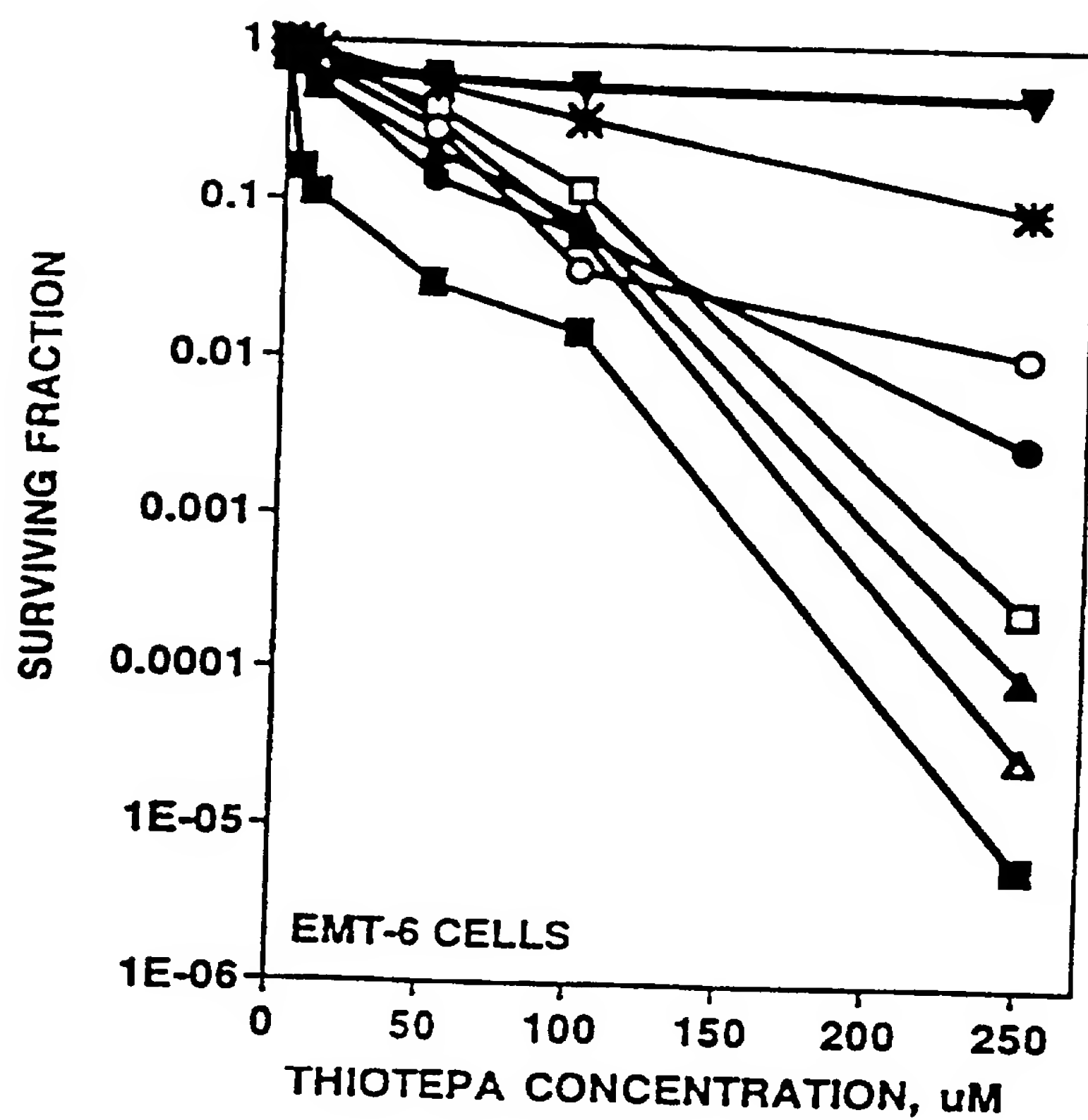
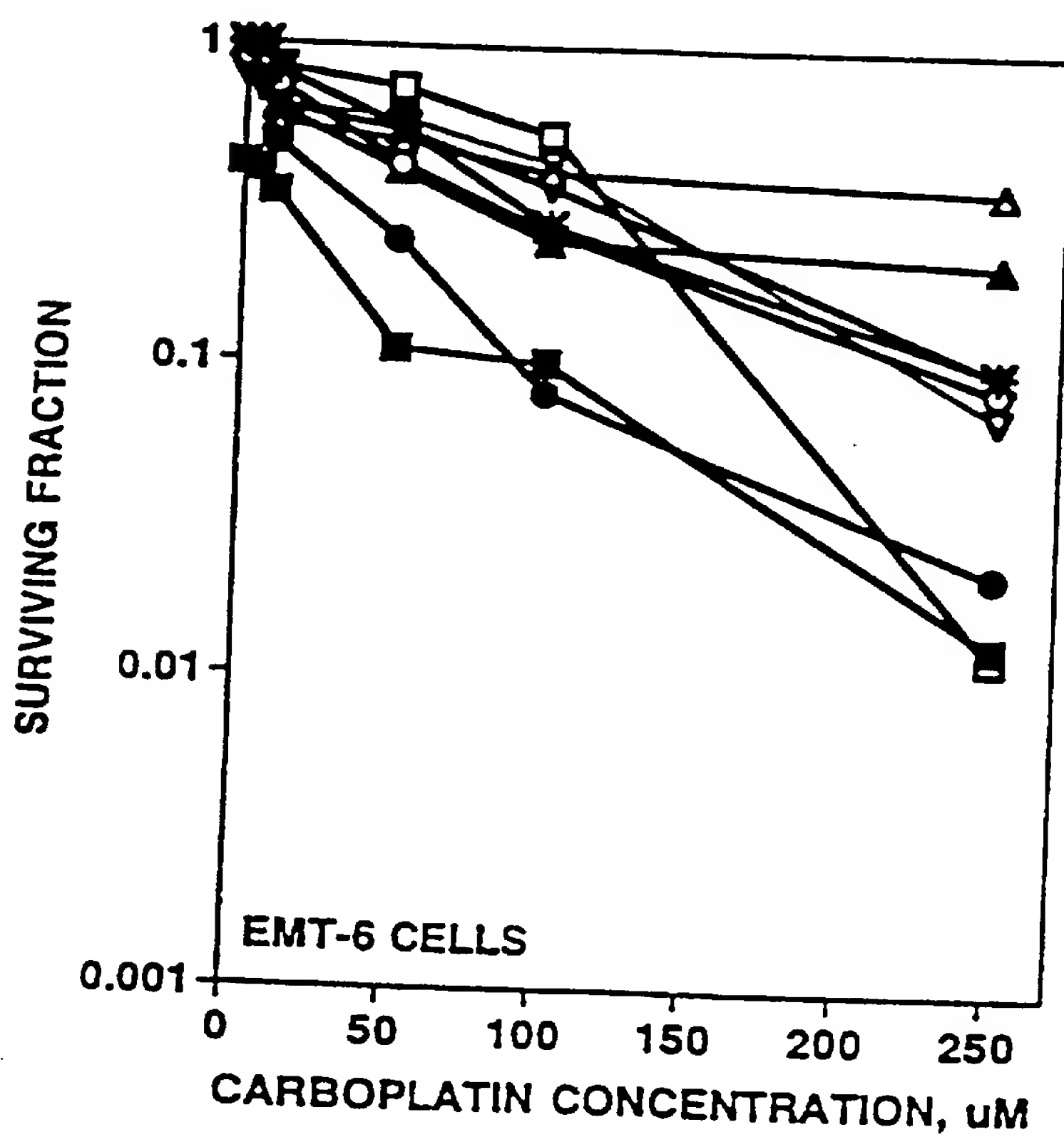


FIG. 7D



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FIG.8A

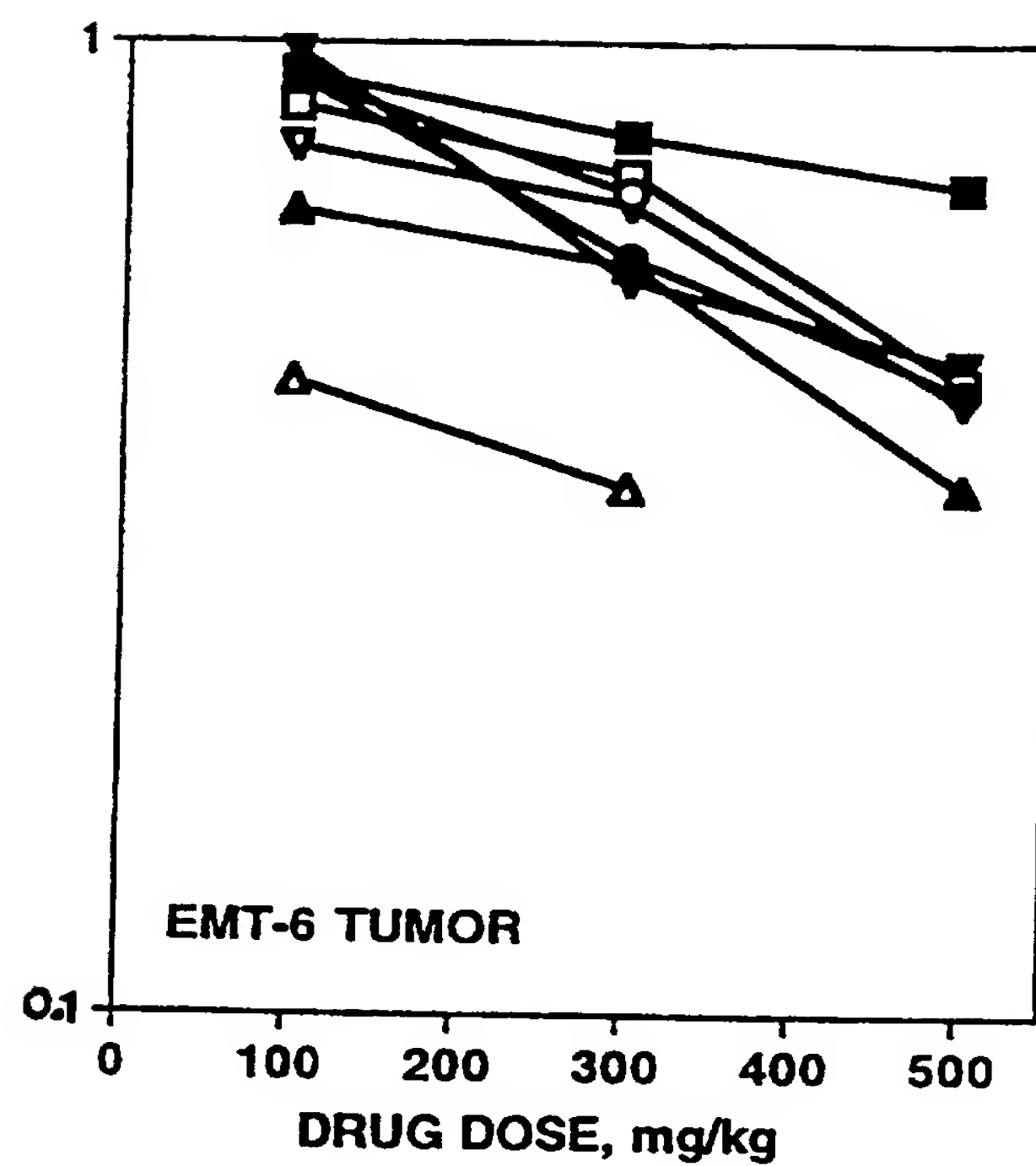
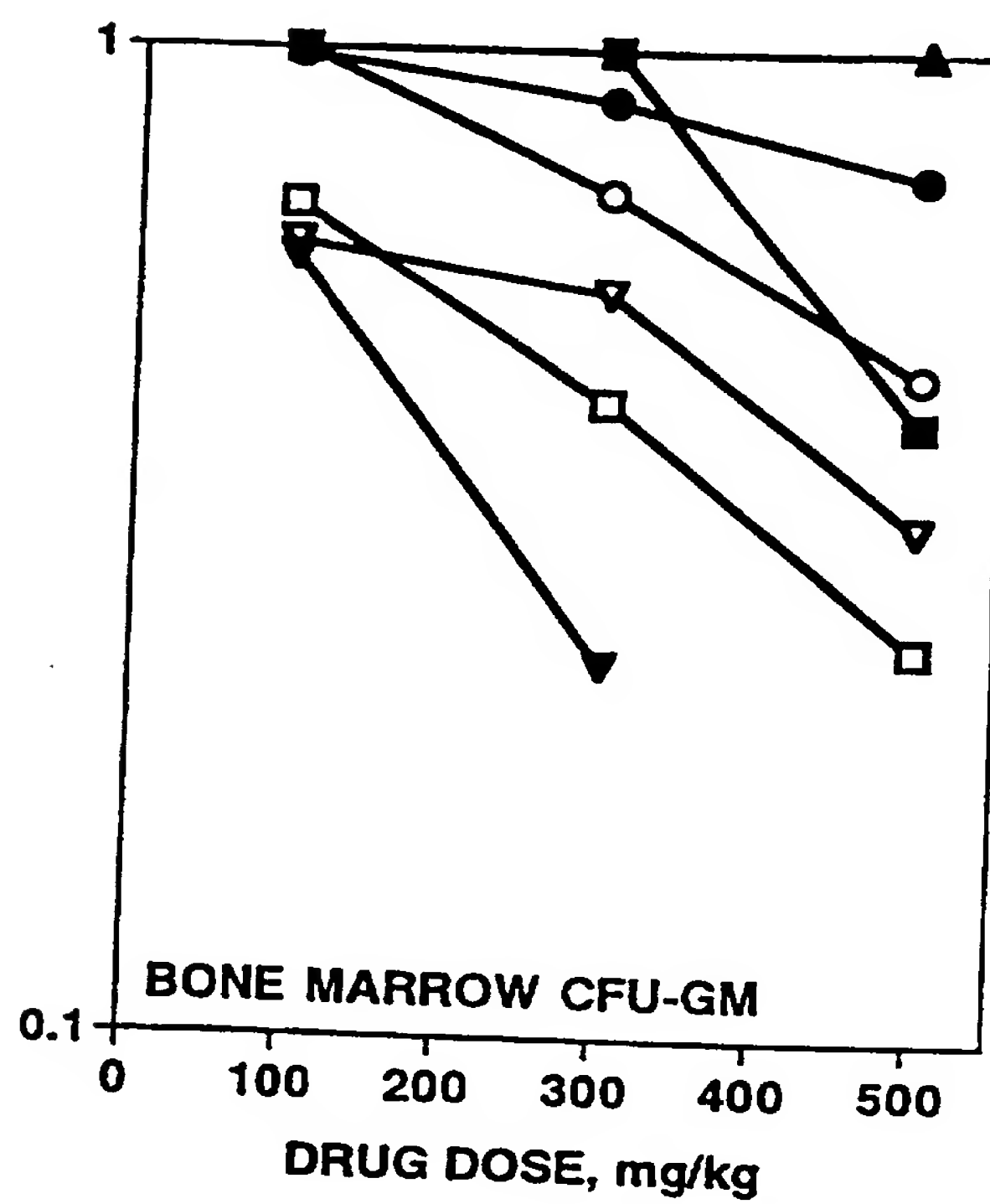


FIG.8B



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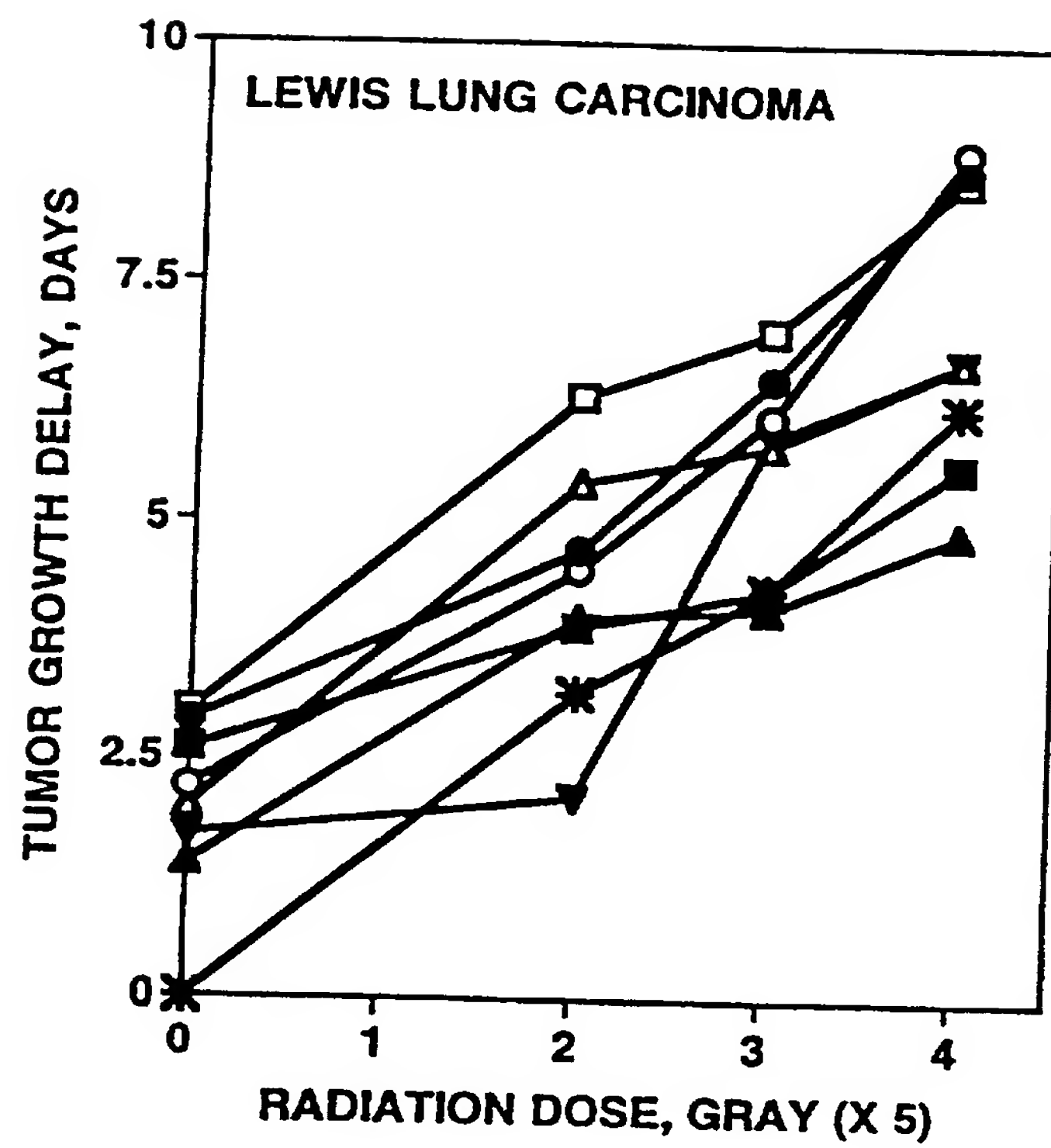


FIG. 9